UNIVERSIDAD DE SEVILLA

FACULTAD DE FARMACIA

EVALUACIÓN DE COMPUESTOS CON ACTIVIDAD BIOLÓGICA EN PRODUCTOS VEGETALES Y BEBIDAS FERMENTADAS. VALIDACIÓN DE MÉTODOS Y APLICACIONES.

MEMORIA PARA OPTAR AL GRADO DE DOCTOR POR LA UNIVERSIDAD DE SEVILLA

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Sevilla, 2018

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INFORMA:

Positivamente para que el doctorando presente la tesis por grupo o compendio de artículos como dispone el artículo 9 de la normativa reguladora de tesis doctoral (Acuerdo 9.1/CG 19-4-12).

Y para que así conste a los efectos oportunos, se firma en Sevilla, a 19 de febrero de 2018.

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Y para que así conste a los efectos oportunos, se firma en Sevilla, a 16 de febrero de 2018.

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AGRADECIMIENTOS

Agradecimientos

Esta Tesis Doctoral se ha realizado en el Área de Nutrición y Bromatología de la Facultad de Farmacia (Universidad de Sevilla) y en el Área de Alimentación y Salud del centro IFAPA-Alameda del Obispo (Córdoba) bajo la dirección de las Dras. Mª del Carmen García Parrilla, Raquel Mª Callejón Fernández y Mª Gema Pereira Caro.

La presente Tesis Doctoral ha sido realizada gracias a la financiación de los siguientes proyectos de investigación:

- Determinación de Melatonina en Uvas, Vinos y Otros Alimentos de Andalucía (P07-AGR-02480) otorgado por la Consejería de Economía, Innovación y Ciencia de la Junta de Andalucía (Investigadora Principal: Dra. Mª del Carmen García Parrilla).

- Evaluación de la Calidad y Seguridad de una Nueva Bebida Obtenida a partir de Fresa no apta para Comercialización (AGL2010-22152-C03-01) otorgado por el Ministerio de Ciencia e Innovación (Investigadora Principal: Dra. Mª del Carmen García Parrilla).

- Caracterización de alimentos y nuevos productos elaborados: potencial saludable, organoléptico y trazabilidad alimentaria. Estrategias de diversificación y reclamo competitivo (PP.AVA.AVA201601.20) otorgado por el Instituto de Investigación y Formación Agraria y Pesquera (Consejería de Agricultura, Pesca y Desarrollo Rural, Junta de Andalucía) (Investigador Principal: Dr. José Manuel Moreno Rojas).

El autor de la Tesis doctoral ha disfrutado de un contrato laboral en el centro IFAPA-Alameda del Obispo (Córdoba) en el marco de las ayudas para la Promoción de Empleo Joven e Implantación de la Garantía Juvenil en I+D+i del Ministerio de Economía y Competitividad.

De las más de 300 páginas que conforman esta tesis, creo que puedo afirmar sin temor a equivocarme que estas han sido las más difíciles de escribir. Los que me conocéis sabéis lo mal que se me dan estas cosas, así que confío en que sabréis disculparme si no logro expresar con palabras lo mucho que os agradezco lo que habéis hecho todos por mí.

En primer lugar, mi agradecimiento más especial para las Dras. Mª Carmen García Parrilla, Raquel Mª Callejón Fernández y Mª Gema Pereira Caro, las directoras de esta Tesis Doctoral. Vuestra ayuda y apoyo han sido imprescindibles para que esta Tesis consiga ver la luz.

A la Dra. Mª Carmen, aún recuerdo tus palabras el primer día que fui al departamento para hacer las prácticas con vuestro grupo, en febrero de 2011. Recuerdo que me pediste que fuera responsable con mi trabajo, espero haber cumplido con las expectativas… Gracias por recibirme con una sonrisa siempre que iba a preguntar algo a tu despacho y por haberme acogido en tu grupo de investigación durante tanto tiempo, por tu confianza y tus consejos.

A la Dra. Raquel, muchas gracias por tu paciencia, por todo el tiempo que me has dedicado y por tu inestimable ayuda en mis peleas con el cromatógrafo. Gracias por enseñarme tantas cosas y por guiarme en mis primeros pasos por el mundo de la investigación. Gran parte de mi trabajo es fruto de tu apoyo y tus consejos.

A la Dra. Gema, tu dedicación, tu apoyo y tus ánimos han sido fundamentales para llegar a donde me encuentro hoy. Gracias por hacerme mucho más fácil la entrada en el intrincado mundo de los polifenoles y, sobre todo, por tu infinita paciencia cada vez que te buscaba para asaltarte con preguntas.

A la Dra. Ana Troncoso, gracias por tu ayuda desinteresada, tu asesoramiento constante y por aportarme siempre grandes ideas.

Al Dr. José Manuel Moreno, gracias por confiar en mí desde el principio, por tu interés en ayudarme siempre en todo lo posible y por brindarme la oportunidad de formar parte de tu grupo de investigación.

También quiero mostrar mi agradecimiento a "mi grupo de Sevilla", del área de Nutrición y Bromatología: Lourdes, Mª Antonia, Ana Belén, Ruth, Edu, Rocío, Cristina y Mabel. Gracias por vuestros ánimos y vuestro apoyo, por vuestra ayuda en el laboratorio, por tantos buenos momentos, por esas charlas durante los almuerzos y, sobre todo, gracias por vuestra amistad. A Félix, gracias por tu alegría, tus consejos y por estar siempre dispuesto a echarme una mano.

A mis compañeros de toxicología, gracias por vuestros ánimos y vuestro apoyo sincero. Por supuesto, no puedo olvidarme de Dani: gracias por tu energía, tu sentido del humor y por esos lunes de fútbol que tan bien nos venían para soltar tensiones.

A “mi grupo de Córdoba”, del área de Alimentación y Salud: Víctor, Curro, María José, Mané, José Carlos, Alicia y Rocío, gracias por vuestro apoyo, por los buenos ratos, por nuestras charlas de futbol, por esas tardes experimentando con cerveza y por conseguir que los días de trabajo sean mucho más entretenidos.

También me gustaría dar las gracias a mis amigos Adri, Juanma, Iván, Javi, Dani, Pilar, Noelia, Juan, Chema, Antonio, Inma, Miguel y alguno más que seguramente me dejaré por ahí. No todo el mundo tiene la suerte de encontrar a sus mejores amigos en la infancia. Yo sí la tuve, y me siento muy afortunado por teneros a mi lado. Gracias por estar siempre ahí, en los buenos y en los malos momentos, por vuestro apoyo incondicional y por tantos años de amistad sin reservas.

A mis compañeros de Biología, mis “Pigs”: Oscar, Juanma, Israel, Rafa, Hansen, Julián, Luis y Pablo, sois la prueba de que la verdadera amistad no entiende de distancia ni de tiempo. Gracias por tantos años de historias memorables (y a veces, ¿por qué no decirlo?, lamentables), por esos reencuentros en los que parece que no ha pasado el tiempo y por hacer que recuerde mi etapa universitaria como una de las mejores de mi vida.

Obviamente, si hay alguien a quien debo darle las gracias es a mi familia: a mi madre, a mi padre, a mi hermano Paco y a mi hermana Ángeles. Gracias por todo lo que me habéis enseñado, por inculcarme los valores del esfuerzo y del trabajo, por apoyarme cuando quise dedicarme a la investigación y por aguantar al niño que sigue estudiando después de tantos años, por soportar mis malos humos cuando esta tesis me estaba volviendo loco y, sobre todo, gracias por ser la mejor familia que uno puede tener. Todo lo que he conseguido y todo lo que soy es gracias a vosotros.

De una forma especial, gracias a Irene, por ser siempre generosa y ayudarme sin pedir nada a cambio nada, por ser mi traductora y obligarte a aprender de aminas biógenas, polifenoles y tantos nombres extraños, por ser tan paciente conmigo, escucharme y estar siempre a mi lado, y por tantas cosas que soy incapaz de describir con palabras…

Y por último, quiero darte las gracias a ti, Dani, porque tú me enseñaste que no importa cuántos obstáculos surjan en el camino, lo importante es luchar siempre con todas tus fuerzas. Te llevo siempre conmigo y te echo de menos, amigo.

Muchas gracias a todos

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RESUMEN

Resumen

La Autoridad Europea de Seguridad Alimentaria (EFSA) es el principal organismo europeo encargado de la Seguridad Alimentaria. En 2011, elaboró una opinión científica sobre la formación de aminas biógenas en alimentos fermentados, en la que se pone de manifiesto la importancia del control de estos compuestos causantes de reacciones adversas en los consumidores. Así, es preciso disponer de mayor conocimiento científico en relación a la presencia de aminas biógenas en alimentos fermentados para realizar una mejor evaluación del riesgo, especialmente en la disponibilidad de métodos de análisis adecuados. Por el contrario, la EFSA también es la encargada de evaluar las Declaraciones de Propiedades Saludables de los alimentos. En este caso, para demostrar una declaración saludable hay que caracterizar el alimento, definir su actividad fisiológica, así como su disponibilidad y plausibilidad biológica. Por todo ello, para poder evaluar las funciones biológicas de los compuestos fenólicos es imprescindible conocer su biodisponibilidad. Además, es importante evaluar e identificar las transformaciones de estos compuestos a lo largo del tracto gastrointestinal, ya que se ha observado que las propiedades bioactivas de los metabolitos, aun siendo los principales implicados en los efectos beneficiosos, suelen ser diferentes a la de sus compuestos originales presentes en los alimentos.

El objetivo de la presente Tesis Doctoral ha sido el desarrollo y validación de nuevos métodos analíticos basados en técnicas cromatográficas para caracterizar y cuantificar compuestos con actividad biológica, como son las aminas biógenas, compuestos fenólicos y sus derivados, en productos vegetales, bebidas fermentadas y muestras biológicas obtenidas de estudios de biodisponibilidad. Estos nuevos métodos han sido aplicados a la resolución de problemas agrupados fundamentalmente en dos grandes bloques temáticos, Seguridad alimentaria y Calidad Alimentaria.

La primera parte de la memoria de esta Tesis doctoral se centró en los principales resultados obtenidos en relación a la determinación de las aminas biógenas, una familia de compuestos que pueden tener efectos perjudiciales para la salud. En primer lugar se realizó un estudio del estado del arte de las técnicas analíticas para la determinación de las aminas biógenas en productos fermentados, realizando una revisión crítica sobre la metodologías empleadas en un periodo comprendido desde 2010 a 2016. Este fue el punto de partida para la posterior adaptación de un método cromatográfico para la determinación simultánea de aminas biógenas y sus precursores (aminoácidos) en la elaboración de una nueva bebida a base de fermentado glucónico de fresa. Con la aplicación de este método se demostró la ausencia de aminas biógenas en los productos finales, resultado seguro al consumidor. Seguidamente, se llevó a cabo una validación de un

método más sensible para la evaluación del perfil de aminas biógenas. Este método fue aplicado para evaluar productos finales (vinagres) o en la conservación de alimentos (vinos abiertos). Las cantidades de aminas biógenas cuantificadas en los vinagres comerciales analizados fueron inferiores a las encontradas en otros productos fermentados, como el vino o el queso, y por tanto no alcanzaron niveles que provoquen efectos perjudiciales para el consumo humano. Además, se controló la evolución de aminas biógenas en botellas abiertas de diferentes vinos durante varios días mantenidas a diferentes condiciones de conservación. En este caso, se observaron ligeros cambios en el perfil de aminas biógenas durante el almacenamiento de las botellas abiertas de vino en las diferentes condiciones.

La segunda parte de esta memoria se centró en la validación de metodologías analíticas para la determinación de metabolitos y catabolitos en muestras biológicas (orina, plasma y heces) tras la ingesta de alimentos ricos en compuestos fenólicos.

Así, primeramente se procedió al desarrollo y la validación de un método para la determinación e identificación de derivados fenólicos en diferentes muestras biológicas (orina, plasma y heces) de ratas tras la ingesta de un extracto de vino rico en proantocianidinas. La aplicación del método permitió identificar por primera vez 8 derivados conjugados de fenilvalerolactonas y 6 derivados de ácidos fenilvaléricos en las muestras de orina, plasma y heces tras la ingesta del extracto de semilla de uva rica en proantocianidinas. Este estudio proporcionó una evaluación detallada de la absorción, el metabolismo y el catabolismo del extracto rico en proantocianidinas por las ratas, mostrando la importante participación de la microbiota colónica en su transformación por el organismo.

Seguidamente, se evaluaron y compararon dos métodos analíticos, un método de cromatografía liquida (HPLC) y un método de cromatografía de gases (GC), ambos acoplados a un detector de masas. Ambos métodos fueron aplicados para la determinación de muestras de orina de voluntarios tras el consumo de zumo de naranja. Además, se evaluó el proceso de extracción comparando la inyección directa y dos extracciones en SPE usando los cartuchos SDB-L y HBL. Del mismo modo, se determinó la eficiencia del proceso de derivatización con *N*-metil-*N*-(trimetilsilil)trifluoroacetamida (MSTFA). Finalmente, aunque ambas metodologías son válidas para el estudio de compuestos fenólicos en muestras de orina, el uso del método de LC permitió obtener una información más detallada de los derivados fenólicos formados del catabolismo de los polifenoles, siendo un método más sensible, menos laborioso y, por tanto, más recomendable que el GC.

INTRODUCCIÓN

Introducción

Los alimentos se definen en el Reglamento (CE) nº178/2002 del Parlamento Europeo y del Consejo de 28 de enero de 2002 como "cualquier sustancia o producto destinado a ser ingerido por los seres humanos o con probabilidad razonable de serlo, tanto si han sido transformados, entera o parcialmente, como si no". De esta forma, dentro de los alimentos se incluyen las bebidas, la goma de mascar y cualquier sustancia, incluida el agua, incorporada voluntariamente al alimento durante su fabricación, preparación o tratamiento.

La demanda de alimentos por parte de los consumidores ha evolucionado en Europa durante la segunda mitad del siglo XX. Así, en la década de los 70, la principal preocupación era garantizar la productividad; en la década de los 80, fue la calidad; mientras que en la de los 90, ha sido la seguridad alimentaria. En la actualidad, este enfoque se ha centrado en garantizar la salud de los consumidores, apareciendo el concepto de alimentos funcionales (Gil, 2017). En la Unión Europea, las Declaraciones Nutricionales y de Propiedades Saludables se encuentran reguladas por el Reglamento (CE) nº1924/2006 del Parlamento Europeo y del Consejo, de 28 de enero de 2002.

En este contexto, para poder evaluar la seguridad y calidad de los alimentos, es importante el empleo de métodos analíticos correctamente validados para determinar compuestos presentes en una matriz de interés. Así, el principal objetivo de la validación de métodos analíticos es definir los requisitos analíticos y confirmar, a través de la aportación de evidencia objetiva, que se han cumplido los requisitos para una aplicación específica y, por tanto, obtener datos de calidad (Eurachem, 2014).

Por todo ello, la investigación de la presente tesis doctoral se ha centrado en la validación de métodos analíticos y la determinación de sustancias de interés, tales como las aminas biógenas y/o aminoácidos, así como los compuestos fenólicos y sus metabolitos, en productos vegetales, fermentados y muestras biológicas, los cuales serán descritos de forma más detallada a continuación.

1. Las Aminas Biógenas

Las aminas biógenas son bases orgánicas nitrogenadas de bajo peso molecular, presentes en el metabolismo normal de los seres vivos (ten Brink et al., 1990; Kirschbaum et al., 1999). Las aminas aparecen de forma natural en los alimentos y se han descrito bajas concentraciones de algunas aminas biógenas de origen endógeno en frutas, verduras, carne, leche y pescado. Sin embargo, se han determinado concentraciones más significativas de estas en productos fermentados, debido a la actividad de diferentes levaduras y bacterias, especialmente, de bacterias lácticas (Önal, 2007). Además, las aminas biógenas son termoestables, por lo que los tratamientos térmicos utilizados en el procesamiento y preparación de alimentos no las inactivan (EFSA, 2011a).

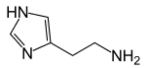
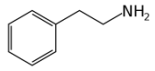
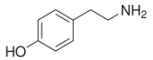
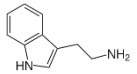
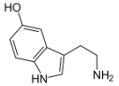
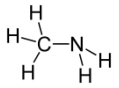
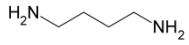
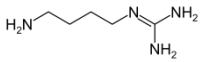
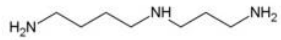
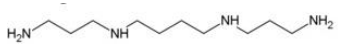
Las aminas biógenas son sintetizadas principalmente por la descarboxilación de aminoácidos y, en menor medida, a través de la aminación y transaminación de aldehídos y cetonas (Askar & Treptow, 1986; Silla Santos, 1996). La formación de aminas biógenas por descarboxilación depende de tres factores principales, como son: el nivel de actividad de las enzimas descarboxilasas, la disponibilidad de aminoácidos como sustrato, así como la presencia o ausencia de la cepa bacteriana específica (Naila et al., 2010). En la **Tabla 1** se pueden observar las principales aminas y sus correspondientes precursores.

1.1. Clasificación de las Aminas Biógenas

Las aminas biógenas pueden clasificarse tanto por su estructura química, como por su volatilidad. En cuanto a su estructura química, se pueden diferenciar en aminas aromáticas y aminas alifáticas (**Tabla 1**). Las aminas aromáticas pueden presentar un núcleo bencénico, como en el caso de la tiramina y la feniletilamina, o pueden poseer un núcleo heterocíclico, como la histamina y la triptamina. Por otro lado, las aminas alifáticas pueden agruparse en dos grupos: monoaminas, como metilamina; y poliaminas, entre las que destacan putrescina, cadaverina, agmatina, espermina y espermidina (Silla Santos, 1996).

Según la volatilidad, las aminas biógenas también pueden clasificarse como no volátiles y volátiles. En general, las investigaciones centradas en aminas volátiles son más escasas que en las no volátiles, con la excepción de la feniletilamina (Almeida et al., 2012). Se ha encontrado una gran diversidad de aminas volátiles en los alimentos, tales como feniletilamina, metilamina, dimetilamina, etilamina, dietilamina, isopropilamina, isobutilamina, amilamina, isoamilamina, pirrolidina, etanolamina y hexilamina, entre otras (Ough et al., 1981; Busto et al., 1995; Peña-Gallego et al., 2012). Por otra parte, entre las principales aminas no volátiles encontradas en los alimentos se incluyen la histamina, la tiramina, la putrescina, la cadaverina, la espermina y la espermidina, la agmatina, la triptamina o la serotonina (Önal et al., 2013).

Tabla 1. Principales aminas biógenas, clasificación y estructura.

Amina biógena	Precursor	Enzima precursora	Clasificación		Estructura
			Estructural	Nº de grupos amino	
Histamina	Histidina	Histidina descarboxilasa	Aromática Núcleo heterocíclico	Diaminas	
Feniletilamina	Fenilalanina	Fenilalanina descarboxilasa	Aromática Núcleo bencénico	Monoaminas	
Tiramina	Tirosina	Tirosina descarboxilasa	Aromática Núcleo bencénico	Monoaminas	
Triptamina	Triptófano	Triptófano descarboxilasa	Aromática Núcleo heterocíclico	Diaminas	
Serotonina	Triptófano	Triptófano monooxigenasa y 5-Hidroxitriptófano descarboxilasa	Aromática Núcleo heterocíclico	Diaminas	
Metilamina	Cetosas Aldehídos	Transaminación	Alifática	Monoaminas	
Putrescina	Ornitina, Agmatina	Ornitina descarboxilasa Arginina deiminasa	Alifática	Diaminas	
Cadaverina	Lisina	Lisina descarboxilasa	Alifática	Diaminas	
Agmatina	Arginina	Arginina descarboxilasa	Alifática	Poliaminas	
Espermidina	Putrescina	Espermidina sintasa	Alifática	Poliaminas	
Espermina	Espermidina	Espermina sintasa	Alifática	Poliaminas	

1.2. Contenido de Aminas Biógenas en alimentos

Tradicionalmente, la presencia de aminas biógenas en los alimentos ha sido un indicador de actividad microbiológica no deseada. De esta forma, se han establecido varios índices de aminas biógenas para evaluar la calidad de los alimentos. Por un lado, se tiene en cuenta la suma de las concentraciones de varias aminas biógenas, principalmente histamina, tiramina, putrescina y cadaverina. En este caso, para los alimentos frescos, los valores menores de 5 mg/kg indican un buen estado, mientras que valores superiores a 20-50 mg/kg muestran un inicio de la degradación del producto. Por otro lado, la relación entre espermidina y espermina también se usa como índice de frescura. Así, una proporción menor de 0.5 indica un producto fresco, mientras que un valor superior a 0.7 está asociado a la descomposición del alimento (Sirocchi et al., 2013; Sentellas et al., 2016).

La presencia de aminas biógenas en los alimentos se debe fundamentalmente a tres razones: están presentes de forma natural, son producto de la contaminación por microorganismos o se forman a partir de procesos de fermentación. Así, su presencia en alimentos está estrechamente relacionada con la actividad microbiológica. De esta forma, la producción de aminas se ha relacionado con la actividad de levaduras y bacterias Gram positiva y Gram negativa (Álvarez & Moreno-Arribas, 2014). Sobre todo, en la familia de las bacterias lácticas se han identificado diferentes rutas de síntesis de estos compuestos, siendo los géneros *Lactobacillus*, *Leuconostoc*, *Pediococcus* y *Oenococcus* los principales productores (Ladero et al., 2010; Linares et al., 2011; Costantini et al., 2013). Además, las aminas biógenas también las sintetizan algunas especies de levaduras o bacterias de la familia *Enterobacteriaceae* (Linares et al., 2012).

Por otro lado, la presencia de ciertas aminas biógenas puede afectar a las propiedades organolépticas de los alimentos. De esta forma, la putrescina se asocia a aromas rancios o desagradables de putrefacción, mientras que la cadaverina confiere a los alimentos olores a carne o vinagre (Sentellas et al., 2016).

En general, las concentraciones más altas de estos compuestos se encuentran en los productos contaminados o fermentados, mostrando unas cantidades significativamente mayores a las presentes en los productos sin fermentar. En la **Tabla 2** se muestra un resumen de las principales aminas biógenas en diferentes alimentos y bebidas. En general, las mayores concentraciones de histamina están presentes en sufu, salsa de pescado, embutidos y queso curado (**Tabla 2**). No obstante, se ha llegado a detectar una concentración de hasta 10000 mg/kg de histamina en pescado en mal estado (EFSA, 2011a). En cuanto a la tiramina, la mayor concentración se ha obtenido en muestras de queso, principalmente, en queso curado (**Tabla 2**). La putrescina y la cadaverina solo se han determinado en cantidades significativas en muestras de carne y pescado ya fermentadas.

Tabla 2. Contenido de las principales aminas biógenas (histamina, tiramina, putrescina y cadaverina) en alimentos y bebidas (mg /kg o mg/L) (Ordóñez et al., 2016).

Producto no fermentado	Histamina	Tiramina	Putrescina	Cadaverina
Verduras				
Repollo	1.00	18.6	11.6	7.6
Soja/tofu	n.d.-5.8	n.d.	0.7-5.0	n.d.-3.4
Frutas y cereales				
Uvas	n.d.-5.8	n.d.-2.4	n.d.-8.0	n.d.-3.4
Manzanas	n.d.	n.d.	5.1	-
Cebada	n.d.	8.0-18.9	7.6-21.0	0.5-1.1
Carnes				
Carne de cerdo	0.9-2.3	n.d.-29.0	n.d.-66.5	1.0-145.4
Carne de pollo	0.2	n.d.-221.6	n.d.-45.9	n.d.-33.5
Carne de ternera	0.4-7.4	n.d.-17.4	n.d.-202.5	n.d.-221.4
Pescados y moluscos				
Anchoas saladas	n.d.-2.0	n.d.-22.3	0.1-8.0	0.1-12.0
Caballa fresca	9.3-12.4	n.d.-0.4	0.2-2.0	1.2-6.6
Atún enlatado	n.d.-110.3	n.d.-48.6	n.d.-116.5	n.d.-103.3
Pulpo	1.3-9.1	n.d.-14.5	2.8-94.1	0.1-164.0
Bebidas				
Leche	n.d.-0.7	n.d.	n.d.	n.d.-0.1
Café preparado	n.d.-1.6	n.d.-19.7	0.4-2.3	0.2-9.1
Zumo de naranja	n.d.-0.04	n.d.-0.06	0.1-2.2	-
Otros				
Miel	n.d.-3.8	n.d.	n.d.	n.d.
Chocolate	0.3-2.0	3.1-8.1	0.8	0.8
Producto fermentado	**Histamina**	**Tiramina**	**Putrescina**	**Cadaverina**
Verduras				
Sauerkraut (chucrut)	2.1-37.0	26.5-94.4	32.1-122	17.1-41.1
Sufu (tofu fermentado)	n.d.-730.0	n.d.-1730	0.5-316.9	0.6-85.8
Salsa de soja	n.d.-85.0	0.9-80.5	7.0-108.0	0.2-85.0
Carne				
Chorizo	n.d.-4.5	3.1-186.1	n.d.-178.3	n.d.-52.0
Embutido	n.d.-514.5	n.d.-509.9	n.d.-505.3	n.d.-689.8
Pescado				
Salsa de pescado	n.d.-729	n.d.-1178	n.d.-1257	n.d.-1429
Productos lácteos				
Yogurt	n.d.-13	n.d.-6.3	n.d.-26.1	n.d.-4.3
Mantequilla	-	4.6-5.0	n.d.	n.d.
Queso sin curar	n.d.-6.67	n.d.-2.74	n.d.-0.9	n.d.-8.9
Queso curado	n.d.-337.9	n.d.-2520	n.d.-105.8	n.d.-774.5
Kéfir de leche	n.d.-4.0	n.d.-9.8	0.4-14.3	n.d.-2.2
Bebidas fermentadas	**Histamina**	**Tiramina**	**Putrescina**	**Cadaverina**
Vino tinto	0.5-27.0	0.1-37.3	2.9-122	n.d.-3.3
Vino blanco	n.d.-3.4	n.d.-6.8	0.8-12.8	n.d.-2.5
Vino de arroz	n.d.-72.1	n.d.-41.4	n.d.-32.3	n.d.-63.5
Cerveza	n.d.-0.3	0.4-5.9	2.1-12.8	0.2-1.4
Vinagre	n.d.-0.3	n.d.-0.2	n.d.-3.2	n.d.-0.1
Sidra	n.d.-6.9	n.d.-5.0	n.d.-12.3	-
Fermentado glucónico	n.d.	n.d.	n.d.	n.d.

n.d.: no detectado; -: no determinado.

La variabilidad del contenido de aminas en alimentos y, especialmente, en vinos y otras bebidas fermentadas, se debe a una gran cantidad de factores, como el tipo de elaboración, el tiempo y modo de almacenamiento, la calidad de la materia prima y la posible contaminación microbiológica durante su elaboración en la bodega (Lonvaud-Funel, 2001). Así, por ejemplo, aunque algunas aminas biógenas se sintetizan tras la fermentación alcohólica, los mayores niveles de aminas biógenas se producen tras la fermentación maloláctica en vinos tintos (Patrignani et al., 2012). Tradicionalmente, la presencia de aminas en los vinos se ha asociado a unas malas condiciones de fermentación y a la contaminación de los mismos. Sin embargo, en un trabajo elaborado por Konakovsky et al. (2011), se observó que las altas concentraciones de algunas aminas en vinos tintos austriacos de alta calidad estaban determinadas por las bacterias seleccionadas para llevar a cabo la fermentación maloláctica durante la elaboración del vino, sin tener malas condiciones higiénicas. En general, la elección de los cultivos iniciales es fundamental para garantizar la calidad del producto final. Recientemente, Berbegal et al. (2017) han logrado aislar cepas autóctonas de *Oenococcus oeni* que llevan a cabo la fermentación maloláctica en vinos tintos de la DO Ribera del Duero sin producir histamina.

Los vinos se han estudiado a lo largo de sus diferentes etapas de elaboración y almacenamiento. De este modo, se ha analizado la concentración de aminas biógenas en uvas (Bauza et al., 2007), mostos (Del Prete et al., 2009; García-Marino et al., 2010; Rodriguez-Naranjo et al., 2013; Wang et al., 2014), fermentación alcohólica (García-Marino et al., 2010; Martínez-Pinilla et al., 2013; Rodriguez-Naranjo et al., 2013; Wang et al., 2014), fermentación maloláctica (García-Marino et al., 2010; Martínez-Pinilla et al., 2013; Wang et al., 2014), envejecimiento en barril o depósitos (García-Marino et al., 2010; Hernández-Orte et al., 2008) y en botella cerrada (González Marco & Ancín Azpilicueta, 2006; Pérez-Magariño et al., 2013). Sin embargo, los ensayos que tratan los cambios que se producen en la concentración de las aminas biógenas en botella abierta son escasos.

1.3. Las funciones biológicas de las Aminas Biógenas

Las aminas biógenas son metabolitos que se encuentran en los tres dominios: arqueas, bacterias y eucariotas (Michael, 2016).

1.3.1. Bacterias

En el caso de las bacterias, estas moléculas tienen diversas funciones, como la producción de energía o el incremento de la resistencia en medios ácidos (Griswold et al., 2006). Así, la descarboxilación de los aminoácidos aumenta la supervivencia en medios ácidos, ya que consume los protones, que junto con la excreción de aminas y CO_2, permiten regular el pH interno (Rhee et al., 2002; Lee et al., 2007). Además, estas moléculas también pueden formar

parte de la producción de energía mediante el uso de bombas electrogénicas antiporte aminoácidos/aminas que generan fuerzas protón-motriz (Molenaar et al., 1993; Wolken et al., 2006). Por otro lado, algunos autores han indicado que ciertas aminas, como la putrescina o la espermidina, actúan frente al estrés oxidativo (Tkachenko et al., 2001; Chattopadhyay et al., 2003) o proporcionando tolerancia al furfural (Geddes et al., 2014). Además, las poliaminas juegan un papel importante en la formación de biopelículas en algunas enterobacterias, como *Yersinia pestis* o *Escherichia coli* (Patel et al., 2006). En cuanto al desarrollo de las bacterias en el tracto gastrointestinal, hay varios autores que han resaltado el importante papel que desempeñan las aminas biógenas. De esta forma, se ha observado que la tiramina interviene en la adhesión de la cepa *Escherichia coli* O157:H7 a la mucosa intestinal (Lyte, 2004). Fernández de Palencia et al. (2011) observaron la importancia de la síntesis de tiramina por *Enterococcus durans* para la adhesión a enterocitos y la reducción de la activación del sistema inmunológico Th1 en el tracto digestivo. Por otro lado, *Lactobacillus brevis* IOEB 9809 produce tiramina y putrescina en respuesta al estrés gástrico, según un ensayo de simulación del tracto digestivo humano (Russo et al., 2012). Además, se ha descrito la importancia de las aminas biógenas en la patogénesis bacteriana de diferentes especies patógenas, tales como *Shigella*, *Streptococcus*, *Salmonella*, *Yersinia*, *Staphylococcus*, *Vibrio*, *Pseudomonas*, *Helicobacter* y *Legionella*, entre otros (Di Martino et al., 2013).

1.3.2. Eucariotas

Respecto a las células eucariotas, la biosíntesis de aminas es importante para la síntesis de hormonas, alcaloides, ácidos nucleicos y proteínas (Premont et al., 2001; Spano et al., 2010).

1.3.2.1. Fungi

Principalmente, se han relacionado las poliaminas con la respuesta al estrés en hongos (Valdés-Santiago & Ruiz-Herrera, 2014). Así, se ha observado que las poliaminas desempeñan un papel importante en la respuesta de los hongos al estrés oxidativo, osmótico o por temperatura (Valdés-Santiago & Ruiz-Herrera, 2014). Por otro lado, existen numerosos estudios que indican la importancia de las poliaminas en la diferenciación celular de hongos, formando parte de la esporulación, germinación de las esporas, colonización y dimorfismo (Valdés-Santiago et al., 2012; Kummasook et al., 2013). Por ejemplo, la espermidina es crucial para la morfogénesis de *Penicillium marneffei* en humanos, ya que permite el crecimiento, la conidiogénesis, la germinación conidial y el dimorfismo. Por todo ello, los autores propusieron la ruta de biosíntesis de esta amina como una diana potencial para combatir infecciones por hongos (Kummasook et al., 2013).

1.3.2.2. Plantas

En plantas, las aminas biógenas están implicadas en varios procesos celulares, incluyendo la división y la diferenciación celular, la síntesis de ácidos nucleicos y proteínas, actuando como estabilizadores de membranas, así como retrasando la senescencia celular, la respuesta a pH y el estrés térmico u osmótico (Li et al., 2006; Handa & Mandoo, 2010; Gupta et al., 2013; Masson et al., 2017). Además, las poliaminas están involucradas en procesos fisiológicos importantes, como el crecimiento y el desarrollo de la fruta (Önal, 2007). Algunos trabajos han sugerido que la acumulación de poliaminas puede tener funciones específicas de protección en plantas adaptadas a ambientes extremos (Li et al., 2006). Además, las poliaminas también tienen importancia en la resistencia de las plantas contra el estrés biótico (Walters, 2003; Jiménez-Bremont et al., 2014), jugando un papel en la resistencia contra hongos (Rodriguez-Kessler et al., 2008) o insectos (Sempruch et al., 2015).

1.3.2.3. Animales

Las aminas biógenas tienen una función importante tanto en la fisiología como en la biología celular en animales. Entre las funciones fisiológicas más importantes reguladas por aminas biógenas, se encuentra la mediación de los primeros síntomas de la respuesta alérgica que lleva a cabo la histamina (Taylor, 1986; Stratton et al., 1991). Así, en ciertos tejidos se libera dicha amina como resultado de hipersensibilidad alérgica o de inflamación (Mahdy & Webster, 2017). La histamina se encuentra de forma natural en la sangre, con una concentración entre 25 y 130 mg/L (Cardona-Galvéz & González-Domínguez, 2005). Del mismo modo, la histamina también participa en la regulación cardiovascular, ya que es un vasodilatador; así como en la regulación de la secreción de ácidos gástricos, a través de receptores H_2 localizados en las células parietales (Silla Santos, 1996, Mahdy & Webster, 2017). Hay una gran variedad de aminas, tales como la histamina, la tiramina, la catecolamina, la dopamina o la serotonina, que funcionan como neurotransmisores en el sistema nervioso central. Además, la histamina y la tiramina pueden actuar como mediadores hormonales en humanos y animales (Bardócz et al., 1995). Asimismo, las aminas también intervienen en la regulación de la temperatura corporal, el control del volumen y pH del estómago y la actividad cerebral (ten Brink et al., 1990).

En cuanto a las poliaminas, se ha observado que son esenciales para un óptimo mantenimiento del funcionamiento normal de la actividad metabólica y del sistema inmunológico del intestino. Además, la espermina y la espermidina parecen estar implicadas en la evolución del tejido intestinal (Ancín-Azpilicueta et al., 2008). Las poliaminas están presentes en los eritrocitos en unas concentraciones de 8–14 y 5–8 nmol/mL para la espermidina y la espermina, respectivamente; mientras que la putrescina no supera el 0.50 nmol/mL (Ducros et al., 2009). A nivel celular, las poliaminas tienen varias funciones como fuente de nitrógeno y como

precursores para la síntesis de hormonas, alcaloides, ácidos nucleicos y proteínas (Silla Santos, 1996). Además, las poliaminas presentan diversas funciones en el mantenimiento de la homeostasis celular, estando implicadas en la estabilización de la membrana celular, la proliferación celular, la síntesis y estabilización de ADN, ARN y proteínas, los factores de crecimiento, los antioxidantes, los reguladores metabólicos, los nutrientes y los mensajeros secundarios (Lozanov et al., 2004; Sánchez-Jiménez et al., 2013). Debido a la diversidad de funciones tanto en el metabolismo celular como en el crecimiento, las poliaminas son necesarias en grandes concentraciones en tejidos de rápido crecimiento (Silla Santos, 1996). Según Bardócz (1995), las poliaminas se pueden considerar como microcomponentes importantes en la alimentación durante periodos de crecimiento intenso de tejidos (maduración del intestino en niños, recuperación postoperativa, etc.). Sin embargo, la recomendación del consumo de fuentes ricas en poliaminas suscita cierta controversia, ya que en los casos en los que se padece alguna patología, como un tumor, el consumo de estas favorecería la proliferación celular, así como el crecimiento y la metástasis de los tumores, favoreciendo la liberación, transporte y colonización de las células cancerosas y evitando la acción del sistema inmune (Kalač & Krausová, 2005; Kalač, 2014; Soda, 2011).

1.4. Efectos Tóxicos de las Aminas Biógenas

Como se ha visto en el apartado anterior, las aminas biógenas son necesarias para distintas funciones en humanos y animales. Sin embargo, la ingesta de alimentos con altas concentraciones de aminas, especialmente histamina y tiramina, puede provocar intoxicación alimentaria, sobre todo en personas sensibles (ten Brink, 1990). En la actualidad, se están desarrollando envases alimentarios que detectan la presencia de aminas biógenas mediante sensores, o que inhiben la formación de estos compuestos (Pacquit et al., 2007; Pereira de Abreu et al., 2012; Heising et al., 2013; Fang et al., 2017).

1.4.1. Histamina

Dentro de las intoxicaciones alimentarias provocadas por aminas biógenas, las más conocidas están causadas por la histamina (Silla Santos, 1996). La histamina es la amina con más efectos tóxicos detectada en alimentos, encontrándose en altas cantidades en pescado, queso, vino y productos cárnicos (ten Brink et al., 1990; Feng et al., 2016). La intoxicación por histamina se define a menudo como "intoxicación por escómbridos", un grupo de pescados donde se encuentran el atún, la caballa y las sardinas. Esta intoxicación se considera un problema mundial (EFSA, 2011a; Naila et al., 2010; Feng et al., 2016). Aunque hay pruebas convincentes para señalar a la histamina como el agente causal de la intoxicación histamínica por pescado, no hay una relación dosis-respuesta simple, ya que el pescado en mal estado con altas cantidades de histamina tiende a ser más tóxico que la misma cantidad de histamina pura administrada por vía

oral (Lehane & Olley, 2000). Por lo tanto, los efectos tóxicos no solo dependen de la concentración de histamina ingerida, sino que también juegan un papel importante la presencia de otras aminas, la actividad de la enzima aminooxidasa y la fisiología intestinal del individuo (Silla Santos, 1996).

La histamina ejerce su efecto tóxico por la interacción de dos tipos de receptores (H_1 y H_2) en membranas celulares. Esta amina tiene una potente acción dilatadora de los vasos sanguíneos periféricos, capilares y arterias, causando una importante caída en la presión sanguínea (Stratton et al., 1991; Ancín-Azpilicueta et al., 2008). Por otro lado, la histamina induce la contracción del musculo liso intestinal, mediado por el receptor H_1, provocando calambres abdominales, diarrea y vómitos (Taylor, 1986). Los síntomas provocados por la histamina son similares a una respuesta alérgica: dificultad para respirar (excitación del musculo liso), dolor de cabeza, rubores faciales, picores, erupciones, inflamación de garganta y fiebre (Stratton et al., 1991; Silla Santos, 1996; Naila et al., 2010). Por otro lado, se han observado otros efectos, como la liberación de neurotransmisores como adrenalina y noradrenalina, la excitación del musculo liso del útero y la estimulación de neuronas sensoriales y motoras (Shabaly, 1996). La **Figura 1** muestra de forma esquemática todos los síntomas que puede provocar la histamina (Maintz & Novak., 2007).

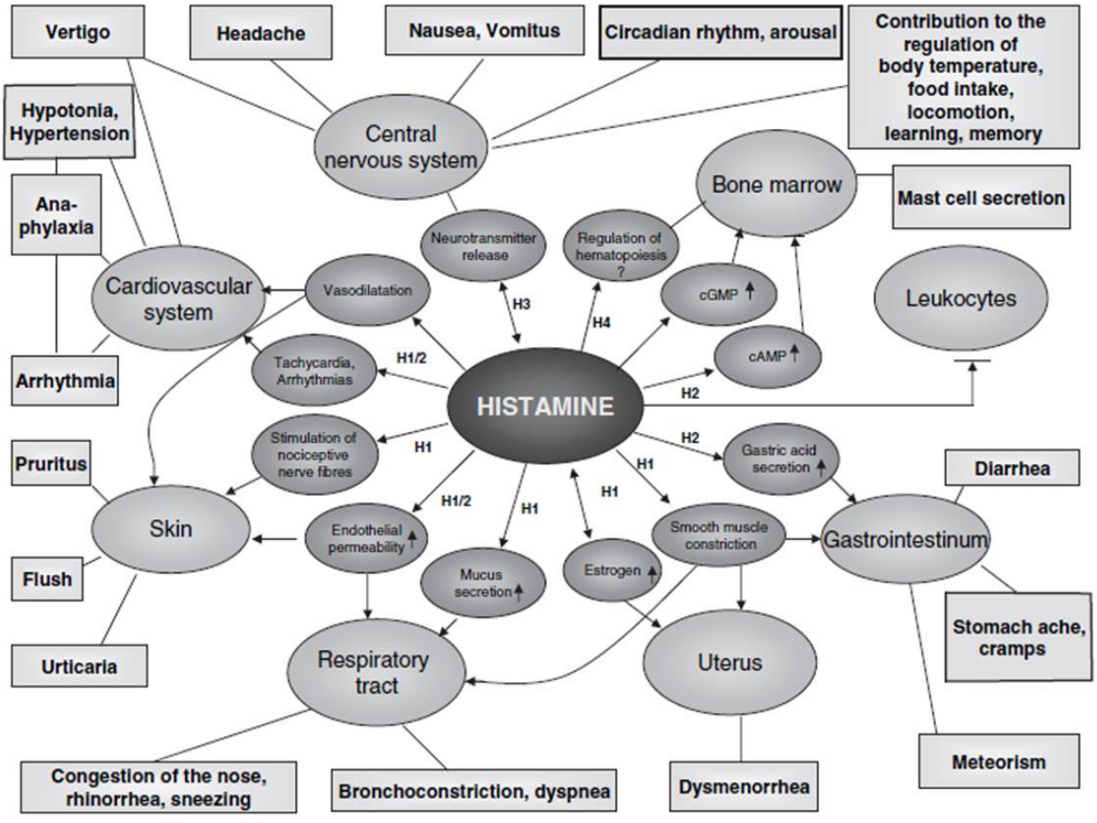


Figura 1. Síntomas provocados por la histamina (Maintz & Novak, 2007).

En cuanto a las cantidades que causan efectos toxicológicos, el consumo de histamina en un rango de concentración entre 8-40 mg, 40-100 mg y mayor de 100 mg puede causar una ligera, intermedia o intensa intoxicación, respectivamente (Ancín- Azpilicueta et al., 2008). Nout (1994) estableció que el nivel máximo permisible de histamina en alimentos debe oscilar entre 50-100 mg/kg. De todos modos, la tolerancia a la histamina dependería principalmente de la sensibilidad de cada consumidor a este compuesto. Una dieta libre de histamina es el tratamiento elegido para los pacientes con intolerancia a la histamina y migrañas, ya que en pequeñas cantidades, puede causar efectos adversos (Jarisch, 2004; EFSA, 2011a).

Respecto al vino, existe cierta controversia acerca de si la histamina es la causa de los casos de efectos tóxicos que se producen por su ingesta. En este sentido, utilizando vinos con alta y baja concentración de histamina, se demuestra que no existe una correlación entre el contenido de dicha amina y la tolerancia de los humanos al vino. Además, parece que la presencia de otros compuestos en el vino, como el acetaldehído, podría explicar el hecho de que haya algunas personas que sean intolerantes a esta bebida, ya que estos podrían provocar la liberación de histamina endógena de los mastocitos e inhibir la enzima DAO, aumentando la concentración de histamina en los tejidos (Zimatkin & Anichtchik, 1999).

1.4.2. Tiramina

En cuanto a la tiramina, esta puede causar una intoxicación conocida como "reacción al queso", con síntomas similares a la intoxicación por histamina (ten Brink et al., 1990). Los efectos farmacológicos debidos al consumo de tiramina son diversos; entre ellos, se encuentran el incremento del gasto cardiaco, lagrimeo, salivación, incremento de la respiración, aumento de los niveles de azúcar en sangre, liberación de noradrenalina en el sistema nervioso simpático (acción vasoconstrictora) y migraña (Shabaly, 1996). En general, los estudios centrados en determinar los niveles de concentración tóxica en humanos obtuvieron datos muy dispares, lo que pone de manifiesto la dificultad para la regulación legislativa de esta amina. Según Askar & Treptow (1986), una dosis de tiramina de 10-80 mg puede causar inflamación tóxica, y más de 100 mg pueden causar migrañas. Sin embargo, Nout (1994) propuso que el nivel máximo permisible de tiramina en alimentos se situase en el rango de 100-800 mg/kg. Aunque tras unos ensayos en ratas, Til et al. (1997) establecieron que el nivel de tiramina sin efectos adversos observables (NOAEL) es 2000 ppm, un informe posterior de la autoridad europea de seguridad alimentaria concluyó que aún no existe información suficiente para establecer el NOAEL en humanos. De esta forma, no se observan efectos adversos por concentraciones de 600 mg en individuos sanos. Sin embargo, los niveles totales de tiramina deben reducirse en pacientes que toman inhibidores de la monoamino oxidasa (MAO), ya que en cantidades mayores a 6 mg de tiramina (si se trata de inhibidores de MAO de primera generación) o 50 mg (en el caso de

inhibidores de MAO de tercera generación), pueden provocar efectos toxicológicos (EFSA, 2011a). Recientemente, Linares et al. (2016) observaron en un ensayo *in vitro* sobre células epiteliales del intestino humano que la citotoxicidad de la tiramina era mayor que la de la histamina. De esta forma, la tiramina provoca necrosis celular, mientras que la histamina induce la apoptosis.

1.4.3. Otras aminas biógenas

En general, la putrescina y la cadaverina tienen menos efectos adversos que la histamina o la tiramina. De esta forma, la putrescina y la cadaverina pueden causar hipotensión, bradicardia, trismo y paresia de las extremidades (Shabaly, 1996). Estas aminas, junto a la espermidina y la espermina, favorecen la absorción intestinal de otras aminas (histamina y tiramina) y disminuyen el catabolismo de estas, y como consecuencia, potencian su toxicidad (Bardócz, 1995). Como en el caso de la tiramina, Til et al. (1997) establecieron un NOAEL para la putrescina y la cadaverina de 2000 ppm; mientras que para la espermidina y la espermina, estos fueron de 1000 ppm y 200 ppm, respectivamente.

En cuanto a la feniletilamina y la triptamina, la información sobre los efectos toxicológicos es más escasa (EFSA, 2011a). Estas aminas, al igual que la tiramina, son vasoconstrictoras y provocan un aumento de la presión sanguínea (Shabaly, 1996; Önal, 2007). En concreto, la feniletilamina es un compuesto volátil que, en cantidades de 3 mg, puede causar migraña (Shabaly, 1996; Ancín-Azpilicueta et al., 2008). La excesiva concentración de feniletilamina en ciertos alimentos, como el chocolate, puede causar trastornos físicos y cefálicos (Ancín-Azpilicueta et al., 2008).

1.4.4. Nitrosaminas

Por otra parte, algunas aminas biógenas, como la agmatina, la espermidina, la espermina o la dimetilamina, pueden reaccionar con ácido nitroso y sus sales formando nitrosaminas, las cuales son moléculas con actividad carcinogénica, mutagénica y teratogénica (EFSA, 2003; Paula Santos et al., 2014). De este modo, estos compuestos pueden afectar al metabolismo mitocondrial, interrumpiendo el equilibrio entre especies reactivas de oxígeno (ROS) y antioxidantes y provocando estrés oxidativo y daño en el ADN (Paula Santos et al., 2014). Las nitrosaminas pueden formarse durante el almacenamiento, la conservación y el cocinado de los alimentos (Silla Santos, 1996). Aunque en vinos apenas hay nitritos (Ough & Crowell, 1980), estos compuestos pueden llegar al intestino a través de otros alimentos, y pueden reaccionar con las aminas presentes en el vino mediante una reacción catalizada por enzimas de la flora intestinal (Allison & McFarlane, 1989).

1.5. Metabolismo y detoxificación de las Aminas Biógenas

Las primeras observaciones sobre la interacción entre el consumo de alimentos con altas concentraciones de aminas y el consumo de fármacos las llevó a cabo un farmacéutico llamado Rowe en 1963. De este modo, observó la reacción que presentaba su esposa, que tomaba el antidepresivo "Parstelin", combinación de tranilcipromina (inhibidor de la MAO) y trifluoperazina, tras consumir queso. Así, describió dos ataques graves en una carta que envió al doctor Blackwell, investigador que estaba evaluando los efectos de la tranilcipromina:

«Quizás le interesen algunos detalles sobre dos graves ataques, similares a aquellos que ha descrito anteriormente, que mi mujer experimentó recientemente durante el tratamiento con "Parstelin". Como soy farmacéutico, tengo un especial interés en el tratamiento.

Primera crisis: después de tomarse una tostada con queso, la cara se le ruborizó a los pocos minutos, sintiéndose muy enferma. El corazón y la cabeza le latían violentamente y el sudor le caía por el cuello. Vomitó varias veces y su estado parecía tan grave que corrí a la calle a buscar a su médico de cabecera. El médico le diagnosticó palpitaciones y recomendó llamarlo si los síntomas no mejoraban en una hora. Finalmente, la gravedad disminuyó y pasadas tres horas, estaba normal, sin más síntomas que una cefalea intensa, pero no de tipo punzante. Ella describió la primera fase de la crisis como si la cabeza le fuera a estallar.

Segunda crisis: Una semana más tarde, de nuevo tras comerse una tostada con queso. Este episodio fue más grave, los vómitos y el dolor de cabeza punzante duraron más allá de las primeras horas de la mañana, e incluso a las 10 de la mañana del día siguiente seguía enferma. Los síntomas disminuyeron en unas 24 horas.

¿Podría haber algún vínculo entre los efectos y los aminoácidos del queso? No hay efectos causados por la mantequilla o la leche. Aunque el tratamiento con "Parstelin" ha continuado, no se han producido más episodios. Si el queso es realmente el factor, tal vez podría explicar la naturaleza esporádica de la incidencia del efecto secundario. Espero que mis comentarios sean de alguna utilidad para sus investigaciones.» (Blackwell et al., 1967).

El tracto intestinal de los mamíferos posee un sistema de detoxificación bastante eficiente, que es capaz de metabolizar la ingesta diaria de aminas biógenas (ten Brink et al., 1990). En condiciones normales, las aminas exógenas absorbidas por los enterocitos desde los alimentos son rápidamente detoxificadas por la acción de amino oxidasas o por conjugación. Las enzimas monoamino oxidasa (MAO) y diamino oxidasa (DAO) juegan un papel importante en el proceso de detoxificación. Además, la histamina también puede ser detoxificada por metilación, mediante histamina N-metiltransferasa (HMT), o por acetilación (Spano et al., 2010). Estas enzimas son inducibles en presencia de monoaminas o poliaminas (Silla Santos, 1996). Por lo

tanto, la intolerancia de ciertos individuos a las aminas biógenas parece estar relacionada con la reducción de la actividad de estas enzimas en el tracto gastrointestinal. Algunas personas pueden tener deficiencias del mecanismo de detoxificación de aminas biógenas debido a razones genéticas, enfermedades gástricas o por la ingesta de fármacos. De esta forma, una actividad insuficiente de la DAO afectaría a la metabolización eficiente de las aminas biógenas, incluso en bajas concentraciones (Önal, 2007). Se ha observado que una concentración elevada de histamina, junto a una disminución de la actividad de DAO, están relacionadas con enfermedades inflamatorias y neoplásicas, como la enfermedad de Crohn, la colitis ulcerosa, la enteropatía alérgica, la alergia alimentaria y los neoplasmas colorrectales (Maintz & Novak, 2007). Los pacientes que usan antidepresivos que inhiben la MAO son más susceptibles a una intoxicación por histamina (Naila et al., 2010; Spano et al., 2010; Önal, 2007) o a padecer una crisis hipertensiva, debido a la acumulación de tiramina en la sangre (Ancín- Azpilicueta et al., 2008). Además de los antidepresivos, existen otros fármacos, como los analgésicos, agentes antimaláricos, diuréticos, antibióticos, mucolíticos y citostáticos, entre otros, que también pueden inhibir las enzimas que metabolizan la histamina (Stratton et al., 1991; Maintz & Novak, 2007; Gillman, 2011). Por otro lado, el consumo de bebidas alcohólicas también provoca el incremento de la sensibilidad hacia las aminas biógenas (ten Brink et al., 1990). Así, el etanol (Ancín-Azpilicueta, 2008) y el acetaldehído (Cardona-Gálvez & Gonzáles-Domínguez, 2005) afectan a la función de estas enzimas.

Como se ha visto en el apartado anterior, los efectos de la histamina y la tiramina pueden verse potenciados por el consumo de otros compuestos, incluyendo otras aminas biógenas como la putrescina y la cadaverina, que tienen una menor actividad farmacológica que la histamina o la tiramina, pero pueden interaccionar con las enzimas amino oxidasas, obstaculizando la actividad de estas (ten Brink et al., 1990; Stratton et al., 1991). De este modo, el sistema de detoxitificación puede verse afectado por las altas concentraciones de ciertas aminas biógenas que se encuentran en alimentos en mal estado o fermentados.

En ensayos con animales, se ha demostrado que la toxicidad de la histamina se incrementa en un factor de 10 cuando se administra 40 minutos después de la ingestión de putrescina y cadaverina (Ancín-Azpilicueta et al., 2008). Otras aminas que también pueden actuar como potenciadores son la tiramina, la triptamina, la feniletilamina, la espermina, la espermidina y la trimetilamina (Stratton et al., 1991; Cardona-Gálvez & Gonzáles-Domínguez, 2005). En concreto, la tiramina actúa sobre la MAO y la triptamina inhibe la DAO, mientras que la feniletilamina actúa inhibiendo la DAO y la HMT (Stratton et al., 1991). En la **Figura 2**, se muestra la diferencia entre la actuación de las enzimas MAO y DAO en condiciones normales, en las que se terminan liberando al torrente sanguíneo productos oxidados inocuos para la salud, y en condiciones en

las que hay una ausencia de actividad enzimática y las aminas con actividad tóxica entran en el torrente sanguíneo.

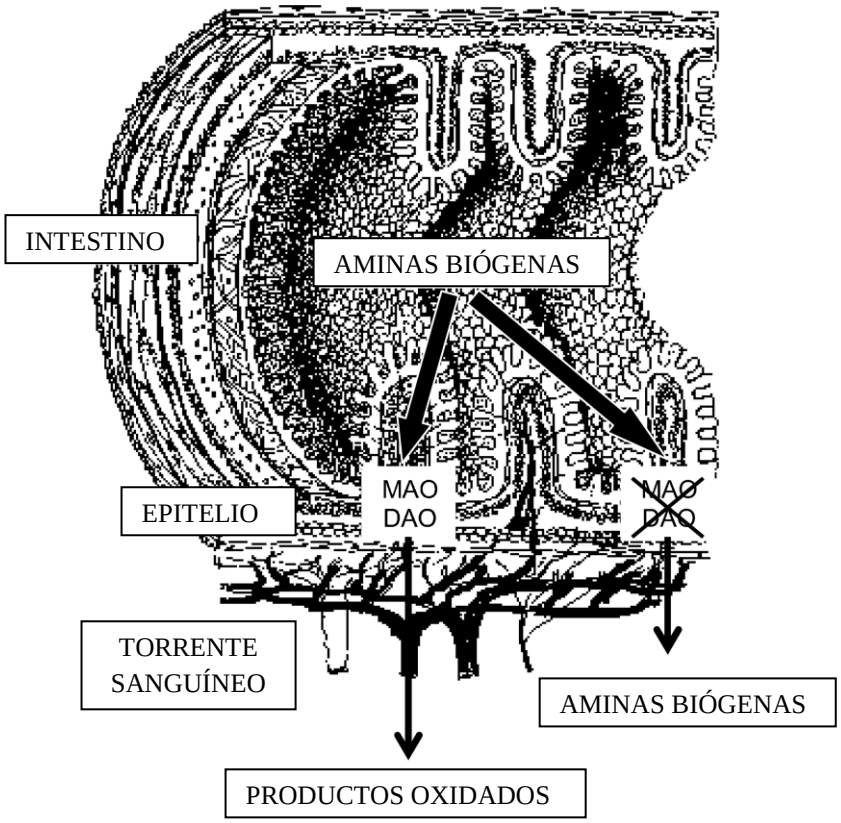


Figura 2. Absorción de aminas biógenas a través del intestino en condiciones normales y en ausencia de actividad enzimática (Ancín-Azpilicueta et al., 2008).

Existen pocos estudios rigurosos sobre los niveles de la actividad DAO gastrointestinal en individuos tolerantes y no tolerantes, o sobre las diferencias que pudieran existir entre los sexos, personas de diferentes edades o incluso personas con diferentes condiciones físicas. No obstante, sí se han identificado los polimorfismos genéticos o diferencias de la actividad DAO entre los individuos tolerantes y los intolerantes (Schewelberger et al., 2003, Petersen et al., 2003).

1.6. Legislación establecida para las Aminas Biógenas en alimentos

Los límites tóxicos de aminas biógenas no se han establecido aún, ya que dependen del tipo de amina, de los alimentos que se ingieran simultáneamente y de la eficiencia del mecanismo de detoxificación de los distintos individuos. En la actualidad, los niveles de histamina están regulados en EE.UU. y en Europa solo para ciertos productos de la pesca, pero no para el vino, el queso y otros alimentos que la contienen (Konakovsky, 2011). Así, la FDA estableció como

límite máximo de histamina una concentración de 50 mg/kg para productos de pesca, mientras que en la Comunidad Europea, este límite está en 100 mg/Kg (Erim, 2013).

La Autoridad Europea de Seguridad Alimentaria (EFSA) ha publicado una opinión científica donde se remarca el riesgo asociado con el incremento de aminas biógenas en los productos fermentados. El documento concluye resaltando la importancia de controlar estos compuestos en los alimentos, así como la necesidad de optimizar y validar los métodos analíticos para diferentes tipos de matrices (EFSA, 2011a).

En este contexto, aunque no se han establecido límites legales para las aminas biógenas, algunos países han propuesto ciertas recomendaciones para los contenidos máximos de histamina, ya que es la amina biógena que provoca el mayor número de casos de intoxicación. Entre estos países, se encuentran Alemania, con 2 mg/L; Bélgica, de 5 a 6 mg/L; y Francia, con 8 mg/L (Bauza et al., 1995; Landete et al., 2005). Por otra parte, Suiza desarrolló un límite legal que no aceptaba vinos con una concentración de histamina mayor de 10 mg/L. No obstante, dicho límite se eliminó en 2008, al ajustarse a la normativa actual de la Unión Europea (Konakovsky, 2011). La Organización Internacional de la Viña y el Vino (OIV) ha publicado, en el compendio de los métodos internacionales de análisis de vinos y mostos, dos métodos cromatográficos para determinar aminas biógenas, a fin de normalizar los métodos de análisis, contribuyendo así a facilitar el comercio internacional (OIV, 2017). Respecto a la cerveza, el *Nutritional codex* de la República Eslovaca recomendó un límite máximo tolerable para la histamina de 20 mg/kg (Karovicova & Kohajdova, 2005).

1.7. Técnicas analíticas para la determinación de Aminas Biógenas

Recientemente, la detección rápida y precisa de bacterias productoras de aminas biógenas en alimentos ha mejorado gracias al empleo de métodos moleculares fiables y rápidos, no dependientes de cultivo, usando principalmente la reacción en cadena de la polimerasa (PCR). De hecho, ha sido posible identificar y cuantificar las bacterias del ácido láctico productoras de aminas en una muestra utilizando varios genes diana. Así, varios autores han afirmado la existencia de una relación entre la presencia del gen que codifica la descarboxilasa y la capacidad de sintetizar aminas biógenas (Fernández et al., 2004; Landete et al., 2005; Lucas et al., 2005; Spano et al., 2010).

Centrándonos en los métodos para la identificación y cuantificación de aminas biógenas y aminoácidos en alimentos, se han empleado varias técnicas analíticas, tales como cromatografía líquida de alta eficacia (HPLC), cromatografía líquida de alta resolución (UPLC), cromatografía de gases (GC), cromatografía de capa fina (TLC), cromatografía líquida de pares de iones (IPLC), electroforesis capilar (CE), sensores y ELISA, entre otros. (Ordóñez et al., 2016;

Sentellas et al., 2016). De todas ellas, la técnica analítica más empleada es la cromatografía líquida usando columnas de fase reversa C18, ya que requiere menos tiempo que otras técnicas y la instrumentación es habitual en el equipamiento de los laboratorios (Peña-Gallego et al., 2012; Hernández-Orte et al., 2003). La detección directa de estos compuestos por HPLC produce interferencias de matriz y además, no tienen buenas propiedades de absorción en el rango de ondas de luz visible, ultravioleta o fluorescencia (Callejón et al., 2008; Peña-Gallego et al., 2009). Por estas razones, la determinación de estos compuestos requiere una derivatización química para mejorar la sensibilidad y la separación, así como para evitar las interferencias de la matriz (Gómez-Alonso et al., 2007; García-Villar et al., 2009; Callejón et al., 2010; Peña-Gallego et al., 2012). En el caso de la detección por espectrometría de masas, la derivatización no es estrictamente necesaria y se pueden desarrollar métodos de detección directa. Sin embargo, la derivación permite reducir la polaridad de las aminas biógenas, mejorando así la separación por las columnas C18 y la sensibilidad en el detector de masas (Erim, 2013).

Los reactivos derivatizantes más utilizados son: Cloruro de dansilo (DnsCl), Cloruro de dabsilo (DbsCl), Cloruro de benzoilo (BCl), ortoftaldehído (OPA), 6-aminoquinolil-N-hidroxisuccinimidil carbamato (AQC) o etoximetilenmalonato de dietilo (DEEMM), entre otros (Callejón et al., 2010; Peña-Gallego et al., 2012).

En la bibliografía se encuentran varios métodos que pueden determinar aminoácidos y aminas biógenas de forma simultánea, usando como reactivo OPA (Kelly et al., 2010; Hu et al., 2014), DnsCl (Mazzucco et al., 2010; Jia et al., 2011; Guo et al., 2015; Tuberoso et al., 2015) o DEEMM (Martínez-Pinilla et al., 2013; Wang et al., 2014). Especialmente, el método propuesto por Gómez-Alonso et al. (2007), que usa como reactivo DEEMM, se ha usado y adaptado para diferentes matrices: vino, cerveza, queso, vinagre… (Martínez-Pinilla et al., 2013; Wang et al., 2014; Redruello et al., 2013; Chinnici et al, 2016). Este reactivo presenta diversas ventajas, ya que puede reaccionar con aminas primarias y secundarias, los derivados aminoenona formados tienen una buena estabilidad, es un reactivo sencillo de usar y no quedan subproductos tras la reacción derivatización (Redruello et al., 2013).

Por el contrario, si el interés de la investigación se centra principalmente en la determinación de las aminas biógenas, la presencia de aminoácidos puede afectar a dicha determinación. Las concentraciones de aminoácidos son uno o dos órdenes de magnitud mayores, y por tanto, consumen un porcentaje significativo del reactivo derivatizante. Una opción es aumentar la concentración de reactivo, aunque no es recomendable, debido a las interferencias que produce el exceso y al aumento de productos de degradación. Otra alternativa es el uso de métodos de extracción, que permiten la concentración de los analitos y la limpieza de las muestras, mejorando la sensibilidad y evitando interferencias (Hernández-Cassou & Saurina, 2011). En

este caso, se han usado distintos tipos de extracción: extracción líquida-líquida (LLE), extracción líquida-líquida asistida por sales (SALLE), extracción en fase sólida (SPE) o microextracción en fase sólida (SPME), entre otras (García-Villar et al., 2009; Mazzucco et al., 2010; Basheer et al., 2011 Ramos et al., 2014).

En el capítulo 1 de la presente memoria se encuentra una revisión pormenorizada y actualizada de las técnicas analíticas empleadas para la determinación de aminas biógenas en bebidas fermentadas desde 2010, resultados ya publicados en la revista *Analytica Chimica Acta* (Ordóñez et al., 2016).

2. Compuestos con actividad beneficiosa. Los Compuestos Fenólicos

2.1. Definición y aspectos generales

Los compuestos fenólicos son un amplio grupo de compuestos orgánicos naturales con la característica estructural principal de que contienen, al menos, un grupo funcional fenol. Estos conforman un grupo heterogéneo, en el que se han descrito más de 8000 distintos, encontrándose en formas libres (minoría), esterificadas o unidos en formas insolubles. Estos últimos suelen estar unidos por enlaces covalentes a compuestos de la pared celular, tales como pectina, celulosa, arabinoxilano y proteínas estructurales. Del mismo modo, los compuestos fenólicos insolubles están presentes en un porcentaje muy variable en muestras de frutas y verduras, con un 24% de media del total de compuestos fenólicos (Acosta-Estrada, et al., 2014).

Los compuestos fenólicos son metabolitos secundarios que se encuentran en hojas, frutos, semillas, tallos, etc. En las plantas, el metabolismo secundario tiene un amplio número de funciones, tales como atrayente para polinizadores y animales dispersores de semillas, protección contra herbívoros, infecciones microbianas, agentes alelopáticos y radiación UV, así como las moléculas señal en la formación de nódulos fijadores de nitrógeno en las leguminosas (Rodríguez-Mateos et al., 2014).

En general, existe una gran cantidad de estudios que han evaluado la capacidad de los compuestos fenólicos como agentes protectores contra varias enfermedades, como enfermedades cardiovasculares, enfermedades neurodegenerativas o cáncer, relacionándolos principalmente con la capacidad de proteger contra daños oxidativos (Del Rio et al., 2013; Rodríguez-Mateos et al., 2014). Además, en los últimos años, se ha visto que estos compuestos también pueden proteger frente a la disminución cognitiva relacionada con la edad, así como las enfermedades neurodegenerativas (Vauzour, 2012). En la **Figura 3**, se observan de forma esquemática los efectos de los compuestos fenólicos sobre la salud.

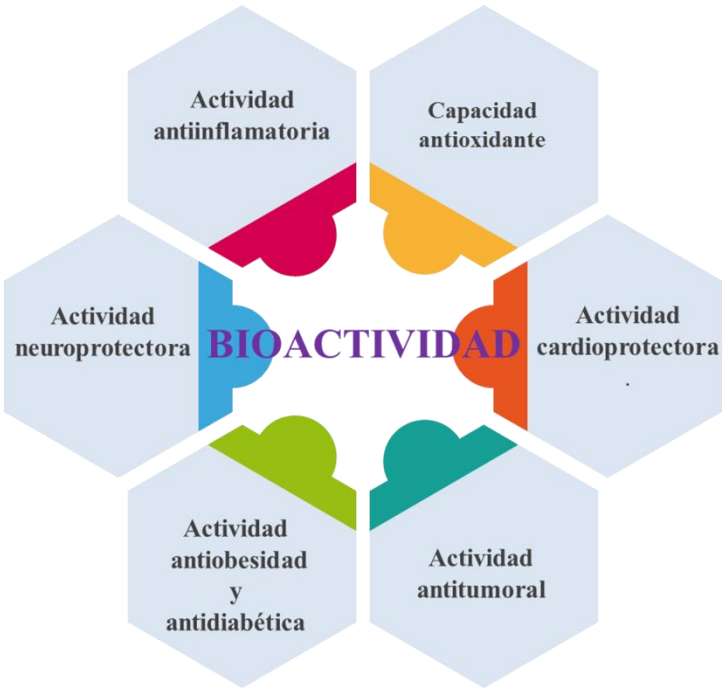


Figura 3. Esquema de los efectos beneficiosos de los compuestos fenólicos.

Por todo ello, existe una evidencia científica relativa a los efectos beneficiosos del consumo de compuestos fenólicos sobre la salud. Sin embargo, en la actualidad no se han establecido protocolos estandarizados, así como el número de estudios necesarios para la evaluación de estos compuestos bioactivos, por lo que las declaraciones sobre las propiedades saludables de la ingesta de estos compuestos son limitadas. En la Unión Europea, la EFSA es el organismo encargado de autorizar las declaraciones saludables en los alimentos, junto con los compuestos con actividad beneficiosa. De esta forma, la EFSA realiza la evaluación de la evidencia científica que soporta las declaraciones, basándose en mecanismos biológicos plausibles que expliquen los efectos saludables y establezcan claramente la relación causa-efecto para poder obtener una evaluación positiva. Además, es necesario que estos efectos se obtengan cuando el alimento que exhibe una declaración sea consumido con un patrón de dieta equilibrada. Por lo tanto, es necesario que los estudios obtengan resultados aceptables y demuestren la plausibilidad biológica de un compuesto bioactivo de forma consistente. Además, se requieren datos de estudios en humanos que aborden la relación entre el consumo del alimento y el efecto, así como que se evalúe un número representativo de la población diana, mientras que los datos en animales o *in vitro* serán incluidos como evidencias de apoyo (EFSA, 2011b). En la actualidad, sólo se han autorizado dos declaraciones de salud relacionadas con la acción de los compuestos fenólicos. Una de ellas está relacionada con los fenoles del aceite de oliva virgen, principalmente referidas el hidroxitirosol y sus derivados (5mg en 20g de aceite), los cuales contribuyen a la protección del LDL frente al estrés oxidativo (EFSA, 2011b). La otra declaración de salud autorizada está relacionada con el consumo de 200 mg al día de los

flavanoles presentes en el cacao, los cuales ayudan a mantener la elasticidad del endotelio que contribuye al flujo normal de la sangre (EFSA, 2012).

2.2. Clasificación y fuentes dietéticas

Los compuestos fenólicos se clasifican atendiendo a su naturaleza química y en función del número de anillos fenólicos, la localización de los sustituyentes y las esterificaciones con azúcares y/o ácidos orgánicos (Strack & Wray, 1989). De esta forma, estos compuestos se pueden dividir en dos grandes grupos, compuestos fenólicos no flavonoides y compuestos fenólicos flavonoides.

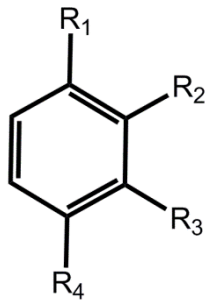
2.2.1. Compuestos fenólicos no flavonoides

Los compuestos no flavonoides son un grupo muy heterogéneo, que incluye desde moléculas sencillas, como los fenoles simples y los ácidos fenólicos, hasta estructuras más complejas, como los taninos hidrolizables. Según sus características estructurales, los compuestos no flavonoides se pueden agrupar en fenoles simples, ácidos fenólicos, alcoholes fenólicos, estilbenos, lignanos y taninos hidrolizables (Bravo, 1998; Scalbert & Williamson, 2000).

En esta memoria nos centraremos en los grupos que han sido determinados: fenoles simples y ácidos fenólicos.

2.2.1.1. Fenoles simples

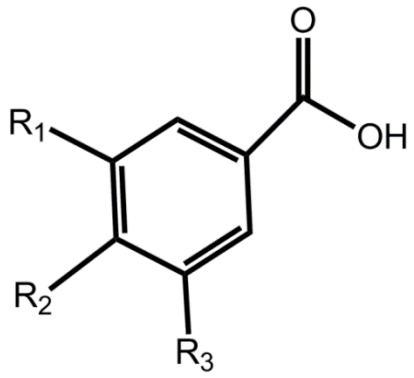
Este grupo está formado por compuestos con una estructura fenólica muy sencilla, conteniendo un solo anillo aromático (C6) (**Figura 4**). Los compuestos que forman parte de este grupo son el fenol, el cresol, el timol y el resorcinol (**Tabla 3**). Estos compuestos se han identificado en diferentes vegetales, como el tomate, el espárrago, el tomillo, el trigo, la avena y el centeno.



Fenoles simples	R_1	R_2	R_3	R_4
Fenol	OH	H	H	H
m-cresol	OH	H	CH_3	H
Timol	OH	H	CH_3	C_3H_6
Resorcinol	OH	H	OH	H

Figura 4. Estructura química de los fenoles simples.

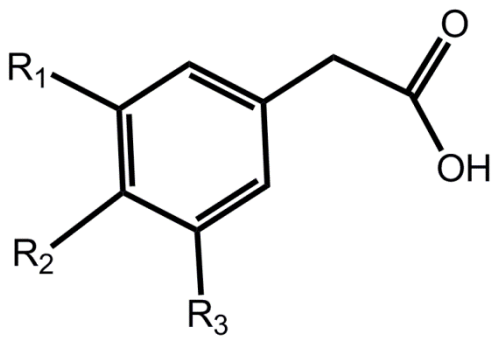
2.2.1.2. Ácidos fenólicos



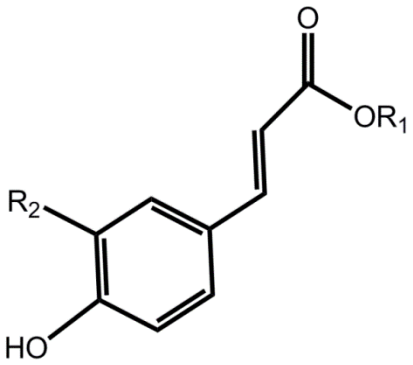
Ác. hidroxibenzoicos	R_1	R_2	R_3
Ác. gálico	OH	OH	OH
Ác. vanílico	OCH_3	OH	H
Ác. p-hidroxibenzoico	H	OH	H
Ác. siringico	OCH_3	OH	OCH_3
Ác. protocatéquico	H	OH	OH

Figura 5. Estructura química de los ácidos hidroxibenzoicos.

Los ácidos fenólicos están formados por un anillo fenólico con un sustituyente de 1 a 3 carbonos. Así, se diferencian los ácidos hidroxibenzoícos (C6-C1), los ácidos fenilacéticos (C6-C2) y los ácidos fenilpropiónicos o ácidos hidroxicinámicos (C6-C3) (**Figuras 5-6**). Los ácidos fenólicos se encuentran en gran cantidad de alimentos con una concentración muy variable. Así, los ácidos hidroxibenzoícos se encuentran principalmente en especias, frutos secos, aceite o té negro; los ácidos fenilacéticos, en cerveza, vino tinto o aceitunas; y los ácidos hidroxicinámicos, en café, tomates y cerveza, entre otros (**Tabla 3**).



Ác. fenilacéticos	R_1	R_2	R_3
Ác. 3-hidroxifenilacético	OH	H	H
Ác. 4-hidroxifenilacético	H	OH	H
Ác. homovanílico	H	OH	OCH_3



Ác. hidroxicinamicos	R_1	R_2
Ác. ferúlico	H	OCH_3
Ác. cafeico	H	OH
Ác. p-cumárico	H	H
Ác. clorogénico	AQ.	OH

Figura 6. Estructura química de los ácidos fenilacéticos y los ácidos hidroxicinámicos. (AQ.: Esterificación con Ácido quínico).

En la **Tabla 3** se encuentran resumidas las principales clases de compuestos fenólicos no flavonoides, los compuestos más característicos y sus fuentes dietéticas.

Tabla 3. Principales clases de compuestos fenólicos no flavonoides (Bravo, 1998; Weichselbaum & Buttriss, 2010; Ignat et al., 2011)

Clase	Subclase	Tipos	Estructura	Compuestos característicos	Fuentes dietéticas
No Flavonoides	*Fenoles simples*		C6	Fenol Cresol Timol Resorcinol	Tomate, Espárrago, Tomillo, Trigo, Avena y Centeno
	Ácidos fenólicos	Ácidos hidroxibenzoicos	C6-C1	Ácido gálico Ácido vanílico Ácido hidroxibenzoico Ácido siringico Ácido protocatéquico	Castaña, Clavo, Té negro, Albahaca, Tomillo, Canela, Cerveza, Nuez moscada, Dátil, Frambuesas, Nueces, Aceitunas, Moras, Arándanos
		Ácidos fenilacéticos	C6-C2	Ác. hidroxifenilacético Ácido homovanílico	Aceitunas, Vino tinto, Cerveza
		Ácidos hidroxicinamicos	C6-C3	Ácido ferúlico Ácido cafeico Ácido *p*-cumárico Ácido clorogénico	Café, Arándanos, Manzana, Sidra, Pera, Melocotones, Cítricos, Salvado de cereales, Espinaca, Brócoli, Col, Tomates, Cerveza, Trigo, Albaricoque, Zanahoria
	Alcoholes fenólicos		C6-C2	Tirosol Hidroxitirosol	Aceite de oliva virgen, Vino tinto y blanco, Cerveza
	Taninos hidrolizables	Elagitaninos	$(C6)_n$	Punicalagina Castalagina	Granada, Fresa, Frambuesa, Nueces, Frutos secos
		Galotaninos	$(C6\text{-}C1)_n$	Ácido tánico	Té, Sidra, Fruta inmadura
	Estilbenos		C6-C2-C6	Resveratrol	Vino tinto, Uva, Bayas, Cacahuetes
	Lignanos		C6-C3-C3-C6	Enterodiol Pinoresinol	Linaza, Semillas de lino, calabaza y sésamo, Avena, Centeno, Trigo, Cebada, Brócoli, Aceite de oliva virgen, Soja

2.2.2. Compuestos fenólicos flavonoides

Los flavonoides son compuestos de bajo peso molecular (15 carbonos) con un esqueleto difenilpropano (C6-C3-C6), compuesto por tres anillos: A y B, anillos fenilo y C, anillo heterocíclico pirano (**Figura 7**). Los principales grupos de flavonoides son: flavonoles, flavonas, flavanoles, flavanonas, antocianidinas e isoflavonas. Además, en la naturaleza se encuentran otros grupos minoritarios, como los dihidroflavonoles, los flavan-3,4-dioles, las chalconas y las dihidrochalconas o auronas.

Los flavonoides se encuentran en la naturaleza principalmente como glicósidos (glucosa, galactosa, xilosa, ramnosa, arabinosa), estando también presentes en menor cantidad en su forma libre (agliconas) o conjugada (esterificados, sulfatados, etc.). La clasificación de los flavonoides suele ser compleja, así que estos compuestos se clasifican según el estado de oxidación del anillo heterocíclico pirano, la posición del anillo fenilo B, el número y posición de los grupos funcionales y la unión a azúcares y ácidos orgánicos, entre otros.

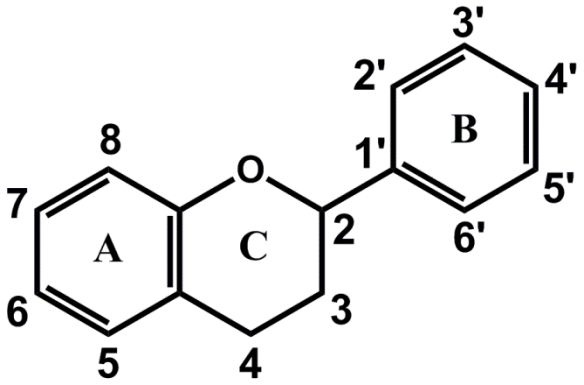


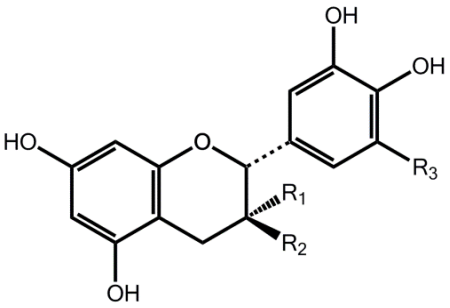
Figura 7. Estructura química general de los compuestos fenólicos flavonoides.

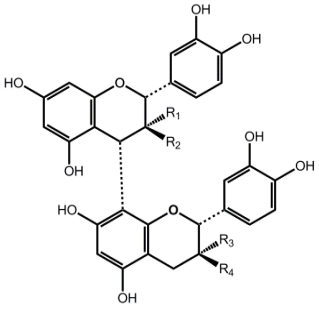
En esta memoria, nos centraremos en los grupos que han sido determinados: flavanoles y flavanonas.

2.2.2.1. Flavanoles

La familia de los flavanoles o flavan-3-oles se caracteriza por tener el anillo pirano saturado y un grupo hidroxilo en el carbono C3 (**Figura 8**). Los compuestos de este grupo son los únicos flavonoides que no aparecen glicosilados en los alimentos, aunque sí suelen encontrarse esterificados, especialmente con el ácido gálico. Los flavanoles se encuentran en forma de monómeros, como catequinas o epicatequinas, o polímeros, como proantocianidinas o taninos condensados. Los monómeros suelen encontrarse en el té, el vino, el cacao y la fruta (**Tabla 4**). Los polímeros se consideran fenoles complejos, aumentando su insolubilidad con el grado de polimerización. Además, pueden formar complejos insolubles con carbohidratos y proteínas,

debido a que su estructura química está altamente hidroxilada. De esta forma, los taninos condensados son los responsables del amargor del chocolate y del carácter astringente de algunas frutas y bebidas (**Tabla 4**) (Scalbert et al., 2005).





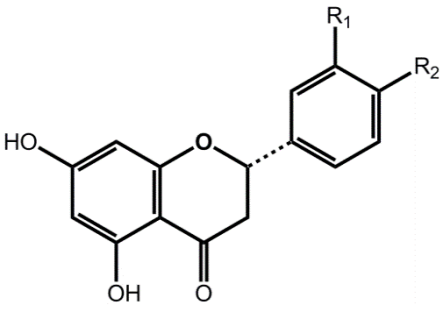
Flavanoles	R_1	R_2	R_3
(+)-Catequina	H	OH	H
(-)-Epicatequina	OH	H	H
Galocatequina	H	OH	OH
Epigalocatequina	OH	H	OH
Epicatequina-3-galato	OH	H	AG.

Proantocianidinas	R_1	R_2	R_3	R_4
Proantocianidina B_1	OH	H	H	OH
Proantocianidina B_2	OH	H	OH	H
Proantocianidina B_3	H	OH	H	OH
Proantocianidina B_4	H	OH	OH	H

Figura 8. Estructura química de los flavanoles (AG.: Esterificación con Ácido gálico).

2.2.2.2. Flavanonas

Las flavanonas tienen el anillo pirano saturado y un grupo cetona en el carbono C4 (**Figura 9**). Estos compuestos se encuentran principalmente en los cítricos, así como en los tomates y las hojas de menta (**Tabla 4**). Los compuestos más destacados son la narirutina y la hesperidina, las cuales son glicósidos de naringenina y hesperetina, respectivamente. Estos compuestos se localizan generalmente en las partes sólidas del fruto, alcanzando una concentración 5 veces mayor que en el zumo.



Flavanonas	R_1	R_2
Naringenina	H	OH
Hesperetina	OH	OCH_3
Eriodictiol	OH	OH

Figura 9. Estructura química de las flavanonas.

En la **Tabla 4**, se encuentran resumidos las principales clases de compuestos fenólicos flavonoides, los compuestos más característicos y sus fuentes dietéticas.

Tabla 4. Principales clases de compuestos fenólicos flavonoides (Bravo, 1998; Weichselbaum & Buttriss, 2010; Ignat et al., 2011)

Clase	Subclase	Tipos	Estructura	Compuestos característicos	Fuentes dietéticas
Flavonoides	*Flavonoles*		C6-C3-C6	Quercetina Kaempferol Miricetina Isoramnetina	Cebolla, Manzana, Pera, Alcaparras, Vino, Té, Uva, Col, Puerros, Brócoli, Apio, Pimienta, Hinojo, Albaricoques, Arándanos, Cacao, Lechuga, Mora, Alforfón, Habas, Tomate, Aceituna
	Flavonas		C6-C3-C6	Apigenina Luteolina	Perejil, Apio, Orégano, Romero, Tomillo, Aceite de oliva, Pimienta, Piel de algunos frutos, Espinaca, Cítricos
	Flavanoles	Monómeros	C6-C3-C6	Catequinas Epicatequinas	Cacao, Vino, Té, Leguminosas, Manzana, Arándanos, Uva, Cebolla, Lechuga
		Taninos condensados	$(C6\text{-}C3\text{-}C6)_n$	Proantocianidinas	Uva, Melocotón, Manzana, Vino, Té, Cerveza, Chocolate, Cacao
	Flavanonas		C6-C3-C6	Naringenina Hesperetina Eriodictiol	Cítricos (Pomelo, Naranja, Limón), Tomate, Menta
	Antocianidinas		C6-C3-C6	Cianidina Pelargonidina Delfinidina Peonidina Petunidina Malvidina	Vino tinto, Uva, Fresas, Moras, Grosellas, Arándanos, Cerezas, Repollo, Frijoles, Cebolla
	Isoflavonas		C6-C3-C6	Daidzeína Genisteína	Soja y sus derivados

2.3. Biodisponibilidad de los compuestos fenólicos

2.3.1. Definición y aspectos generales

El estudio de la biodisponibilidad de un compuesto engloba el análisis de su absorción, distribución, metabolismo y excreción en la matriz alimentaria en la que se encuentre (ADME) (Kay et al., 2016), resultando primordial para poder establecer su actividad biológica, así como sus potenciales efectos beneficiosos para la salud (Kroon et al., 2004). La bioactividad está definida como la capacidad de un compuesto de interactuar con una diana biológica y producir una respuesta. Así, la bioactividad depende del proceso ADME para que se produzca esta interacción, ya que es necesario que llegue al órgano diana englobando la absorción y las transformaciones en diversos metabolitos (Porrini & Riso, 2008; Fernández-García et al., 2009). Entre los factores que influyen en la absorción de un compuesto se puede resaltar la estructura química de este, ya que es el factor principal que determina su absorción (Scalbert et al., 2002). No obstante, existen otros factores de origen genético, ambiental y culinario que podrían afectar a su absorción y posterior metabolización en el organismo.

La mayor parte de los compuestos fenólicos presentes en los alimentos no se encuentran libres en su forma nativa, sino unidos a grupos acilos, esteres, glicósidos o incluso unidos a otros compuestos fenólicos formando estructuras más complejas. Tras la ingesta de alimentos, aunque la actividad hidrolítica de la saliva puede modificar los compuestos fenólicos en su forma nativa conjugada en la cavidad oral, la mayoría de ellos son estables en las condiciones hidrolíticas que actúan tanto a nivel oral como estomacal, alcanzando el intestino delgado en su forma acilada, esterificada, glicosilada o incluso polimerizada (Rodríguez-Mateos et al., 2014).

En el intestino delgado, el tamaño de las partículas de los alimentos se reduce, mejorando la liberación de los compuestos fenólicos al lumen intestinal y permitiendo su modificación por la acción de enzimas digestivas (Scalbert et al., 2002). En general, los compuestos fenólicos acilados pueden ser absorbidos sin hidrólisis previa a través de los transportadores de monocarboxilato (MCT) (Konishi et al., 2006; Lafay & Gil-Izquierdo, 2008). Del mismo modo, Novotny et al. (2012) observó que la vida media en plasma de las antocianinas aciladas era menor que la de las no aciladas, mostrando que su absorción tiene lugar en la primera etapa del tracto intestinal.

Los compuestos fenólicos glicosilados pueden sufrir la escisión del azúcar en el lumen, generalmente mediada por la acción de la lactasa-floricina hidrolasa (LPH), enzima que se encuentra en el borde en cepillo formado por las microvellosidades de las células epiteliales. En particular, la enzima LPH presenta una alta especificidad para los flavonoides-*O*-β-*D*-glucósidos, liberando las agliconas, que pueden entrar en las células epiteliales por difusión pasiva, debido a que aumenta su capacidad lipofílica (Day et al., 2000). Además, se ha descrito

que la β-glucosidasa citosólica (CBG) también puede actuar fragmentando el grupo glicósido de los compuestos fenólicos. Para la absorción en el enterocito de los compuestos fenólicos, se necesita el transporte activo hacia el interior de este, el cual posiblemente esté mediado por el transportador dependiente de glucosa, SGLT-1 (Gee et al., 2000). Del mismo modo, las proteínas facilitadoras del transporte de glucosa 2 (GLUT2) parecen intervenir en la absorción de compuestos fenólicos, concretamente de las antocianinas, en el intestino delgado (Faria et al., 2009).

Por otro lado, la presencia de enzimas esterasas en el intestino delgado, tanto luminales como en células de la mucosa intestinal, permite la hidrólisis de los compuestos esterificados, por lo que pueden liberarse algunos ácidos hidroxicinámicos para su posterior absorción intestinal (Andreasen et al., 2001).

La absorción también está directamente relacionada con el peso molecular y el grado de polimerización. De esta forma, los monómeros e incluso dímeros de flavanoles, aunque en limitada proporción, pueden absorberse en el intestino delgado, mientras que los polímeros más grandes, como las proantocianidinas, no pueden atravesar la barrera intestinal (Ou & Gu, 2013).

Los compuestos fenólicos que se absorben en el intestino delgado sufren una extensa transformación por la acción del metabolismo enzimático de fase II. Estas enzimas llevan a cabo reacciones de conjugación sobre las agliconas con el ácido glucurónico, catalizado por las UDP-glucuronosiltransferasas (UGT) endoplasmáticas; con grupos sulfato, catalizados por las sulfotransferasas (SULT) citosólicas; o grupos metilo, catalizados por la enzima catecol-*O*-metiltransferasa (COMT) (Del Rio et al., 2013). Por lo tanto, el metabolismo catalizado por las enzimas de la fase II conduce a derivados glucuronidados, sulfatados, metilados o incluso combinaciones de estos, como derivados diglucurónidos, disulfatados, metilsulfatados, metilglucurónidos y sulfoglucurónidos, que se llevan a cabo principalmente en los enterocitos del intestino delgado y en el hígado y son posteriormente eliminados por excreción renal. Parece que existe la posibilidad de que los metabolitos de fase II vuelvan al intestino delgado a través de la bilis por de la recirculación enterohepática (Actis-Goretta et al., 2013; Crozier, 2013). Alternativamente, los metabolitos de fase II también pueden volver de nuevo al lumen desde el intestino delgado por mediación de la familia de proteínas transportadoras de membrana dependientes de ATP (ABC) (van de Wetering et al., 2009; Manzano & Williamson, 2010). En general, los metabolitos conjugados en el enterocito pasan al torrente sanguíneo, donde serán distribuidos por los tejidos y finalmente, eliminados en la orina.

Los estudios de biodisponibilidad de los compuestos fenólicos han demostrado que algunos de estos compuestos son absorbidos y metabolizados en gran proporción en el intestino. Un claro ejemplo es el ácido gálico, que se absorbe en torno a un 40 % tras su ingesta (Manach et al.,

2005). Sin embargo, en investigaciones realizadas con voluntarios con ileostomía, se ha demostrado que muy pocas estructuras fenólicas pueden absorberse en el intestino delgado, por lo que la mayoría de estos compuestos, en torno a un 90 %, pasan de forma inalterada al colon (Dufour et al., 2018). Así, la mayoría de los compuestos fenólicos presentan una baja o limitada biodisponibilidad a nivel del intestino delgado, como es el caso de las flavanonas o proantocianidinas, que alcanzan el intestino grueso, donde se transformarán por la acción de la microbiota intestinal (Jaganath et al., 2006; Stalmach et al., 2009, 2010; Borges et al., 2013; Erk et al., 2014). Las bacterias intestinales actúan hidrolizando los conjugados (glucósidos, glucurónidos, sulfatos, amidas, ésteres, polímeros y lactonas) y fraccionando el esqueleto de los compuestos fenólicos, así como la fisión de los anillos fenólicos, mediante reacciones de reducción, descarboxilación, desmetilación y deshidroxilación (Selma et al., 2009). Estas modificaciones estructurales transforman los compuestos fenólicos más complejos en formas químicas más sencillas de menor peso molecular, formando los llamados catabolitos, como los ácidos fenólicos, que pueden absorberse pasando alguno de ellos al metabolismo fase II (los colonocitos o el hígado) antes de ir a la recirculación sistémica. Los catabolitos se absorben y pasan a la circulación sistémica, siendo distribuidos a los tejidos y, finalmente, excretándose en la orina (Crozier et al., 2010). Por otro lado, parte del carbono de los glucósidos y del anillo A de los compuestos fenólicos puede metabolizarse como ácidos grasos de cadena corta, pasando al metabolismo energético del huésped (Czank et al., 2013).

Adicionalmente, unos estudios recientes han demostrado que si se tienen en cuenta los metabolitos procedentes de la absorción en el intestino delgado (derivados glucurónidos, metilados y sulfatados) y los catabolitos formados a nivel del intestino grueso, los compuestos fenólicos son mucho más biodisponibles de lo que se pensaba hace unos años (Rodríguez-Mateos et al., 2014; Pereira-Caro et al., 2014; Kay et al., 2017). Por lo tanto, durante los últimos años se está poniendo de manifiesto el papel tan importante de la microbiota intestinal en la biodisponibilidad de los compuestos fenólicos (Williamson & Clifford, 2017). En este sentido, el metabolismo de la microbiota intestinal modula los efectos sobre la salud de los compuestos fenólicos, ya que afecta a su biodisponibilidad y, por lo tanto, a su actividad biológica potencial (Ludwig et al., 2013). Además, se ha demostrado que las propiedades bioactivas de los metabolitos y catabolitos microbianos suelen ser diferentes a las de sus compuestos originales (Duda-Chodak et al., 2015). De esta forma, los catabolitos fenólicos son los compuestos principalmente implicados en los efectos beneficiosos, como la actividad antioxidante, antiinflamatoria, antihiperglicemica o neuroprotectora (Verzelloni et al., 2011; Masella et al., 2012; Duda-Chodak et al., 2015). La dieta puede afectar a la composición de las poblaciones microbianas presentes en el intestino, y por lo tanto, afecta a la actividad metabólica de éstas (Selma et al., 2009; Duda-Chodak et al., 2015). En general, existe una gran variabilidad en la

absorción de los compuestos fenólicos, la cual es el resultado de la interacción entre los alimentos consumidos, la microbiota intestinal y el huésped (Williamson & Clifford, 2017).

2.3.2. Biodisponibilidad de flavanoles

Hay una gran cantidad de estudios sobre la absorción y el metabolismo de los flavanoles en humanos. En general, se ha observado que en torno a un 30 % de los monómeros y ciertos dímeros de flavanoles se absorben en el intestino delgado por difusión pasiva (Manach et al., 2005; Borges et al., 2010). Una vez absorbidos, son ampliamente metabolizados, sufriendo procesos de conjugación con grupos sulfatos, glucurónidos o metilados. Concretamente, tras el consumo de 500 ml de té verde con una concentración de 648 µmoles de flavanoles, se encontraron hasta 12 metabolitos en plasma en forma de conjugados O-metilados, sulfatados y glucurónidos de (epi)catequina y (epi)galocatequina, siendo el compuesto principal la (epi)galocatequina-O-glucurónido. La concentración plasmática máxima encontrada de éstos metabolitos se dio 2 horas tras su ingesta, lo cual indica que su absorción en forma de agliconas tiene lugar principalmente en el intestino delgado, siendo posteriormente metabolizadas a sus formas conjugadas (Del Río et al., 2013). Además, se determinaron otros compuestos sin transformación, como (-) -epigalocatequina-3-*O*-galato y (-) -epicaquina-3-*O*-galato. Aunque la absorción directa de flavonoides no es muy común, el éster de galato presente en estos compuestos podría favorecer su absorción.

Recientemente, Ottaviani et al. (2016) realizaron un seguimiento del consumo de 60 mg de (-)-epicatequina [2-^{14}C] marcada en 8 voluntarios, determinando que alrededor del 82 ± 5% del total ingerido era absorbido y extensamente metabolizado e identificando más de 20 metabolitos derivados en orina. Los principales metabolitos encontrados fueron la (-)-epicatequina-3'-*O*-glucuronido, (-)-epicatequina-3'-*O*-sulfato, 3'-*O*-metil-(-)-epicatequina-5-sulfato y 3'-*O*-metil-(-)-epicatecatequina-7-sulfato (Ottaviani et al., 2016). Del mismo modo, se observó que alrededor del 20% se absorbió en el intestino delgado, mientras que el resto pasó al colon, donde se transformó por la acción de la microbiota, produciendo un amplio abanico de catabolitos, como los derivados de γ-valerolactona y γ-hidroxivalerolactona y derivados del ácido hipúrico y ácidos fenólicos.

Actis-Goretta et al. (2013) observaron que en torno al 44 % de los monómeros de flavanoles alcanzan el colon sin haber sido absorbidos ni metabolizados. Sin embargo en unos estudios realizados en voluntarios con ileostomía, esta cantidad aumentó a valores superiores al 100 % de los monómeros consumidos, siendo la mayoría derivados sulfatados y metil-sulfatados (Borges et al., 2013). Del mismo modo, el 22 % de los dímeros (procianidina B1 y B2) se recuperaron en el fluido ileal (Borges et al., 2013). Así, en el colon, la microbiota intestinal transforma tanto los monómeros como los dímeros de flavanoles en ácidos fenólicos,

fenilvalerolactonas y ácidos fenilvaléricos (Ottaviani et al., 2016; Borges et al., 2017). Varios estudios centrados en el metabolismo microbiano intestinal tras el consumo de té verde han identificado una gran cantidad de este grupo de metabolitos en la orina, así como conjugados de ácidos valérico y fenilvalérico, excretados entre 5 y 10 horas tras la ingesta. Los resultados mostraron una alta variabilidad en la relación absorción/excreción de los metabolitos de valerolactona entre los sujetos (Calani et al., 2012; van der Hooft et al., 2012).

En la **Figura 10**, se puede observar una representación esquemática de los principales metabolitos y catabolitos excretados en orina en función del tiempo tras la ingesta de la (-) – epicatequina en humanos.

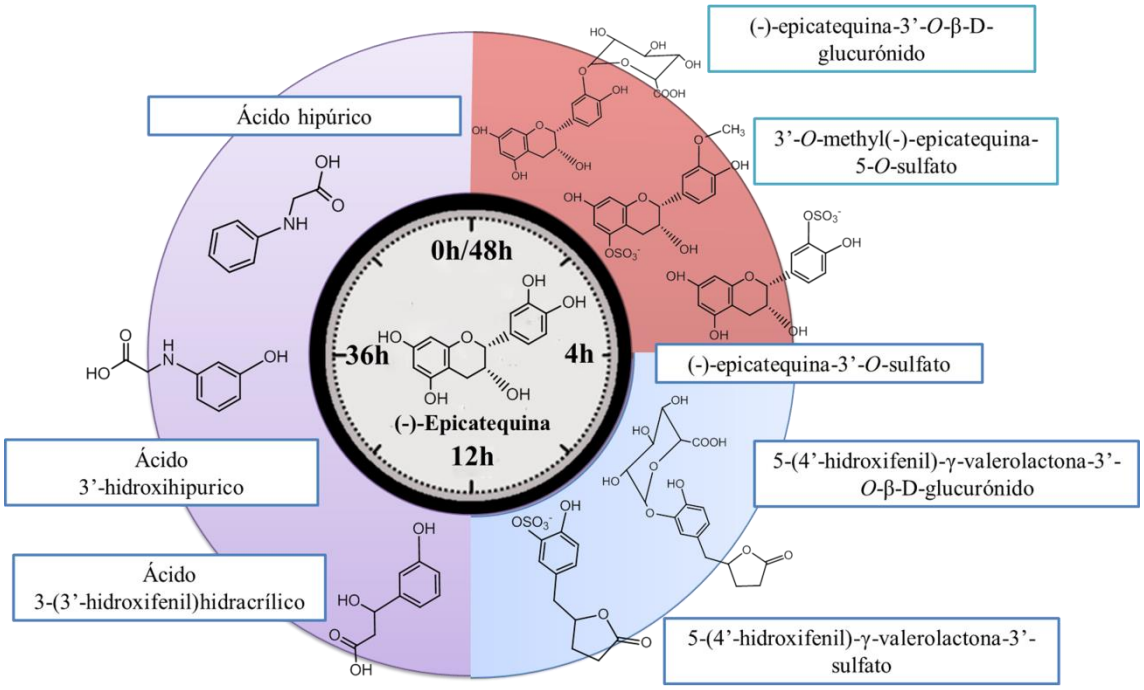


Figura 10. Representación esquemática del metabolismo de la (-) - epicatequina en la orina en humanos en función del tiempo (modificación de Ottaviani et al., 2016).

En general, los principales metabolitos de epicatequina absorbidos en el intestino delgado y derivados de las enzimas presentes en los enterocitos y hepatocitos encontrados en plasma y orina son (-)-epicatequina-3'-*O*-glucurónido, (-)-epicatequina-3'-*O*-sulfato, 3'-*O*-metil-(-)-epicatequina-5-sulfato y 3'-*O*-metil-(-)-epicatecatequina-7-sulfato (**Figura 11**). Por el contrario, los catabolitos producto de la microbiota colónica se absorben en el intestino grueso y son transformados por las enzimas de los colonocitos y hepatocitos. Los compuestos principales encontrados en plasma son 5-(4'-hidroxifenil)-γ-valerolactona-3'-sulfato, 5-(4'-hidroxifenil)-γ-valerolactona-3'-*O*-glucurónido, 5-(3'-hidroxifenil)-γ-valerolactona-4'-*O*-glucurónido, ácido 3-3'-hidroxifenilpropionico, ácido hipúrico y ácido 3'-hidroxihipurico (**Figura 12**).

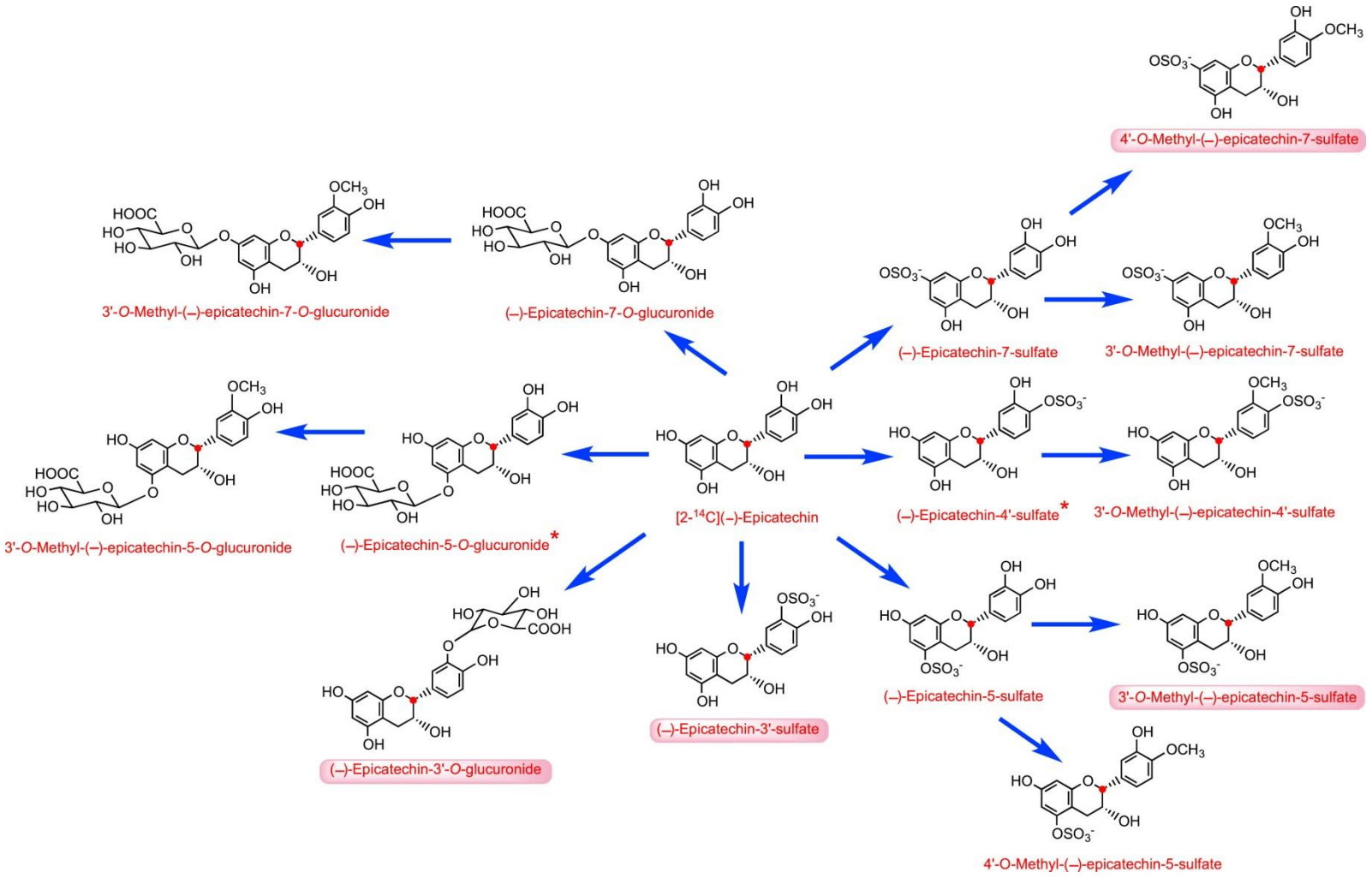


Figura 11. Rutas propuestas para el metabolismo humano de (-)-epicatequina, potencialmente en enterocitos y hepatocitos, después de su ingestión en el intestino delgado. Los nombres de metabolitos en los recuadros indican los principales productos que se acumulan en el plasma y la orina después de la ingesta de (-) - epicatequina. Las flechas azules indican las conversiones que son catalizadas por enzimas de mamíferos. Los asteriscos indican intermedios potenciales que no se acumulan en cantidades detectables (Borges et al., 2017).

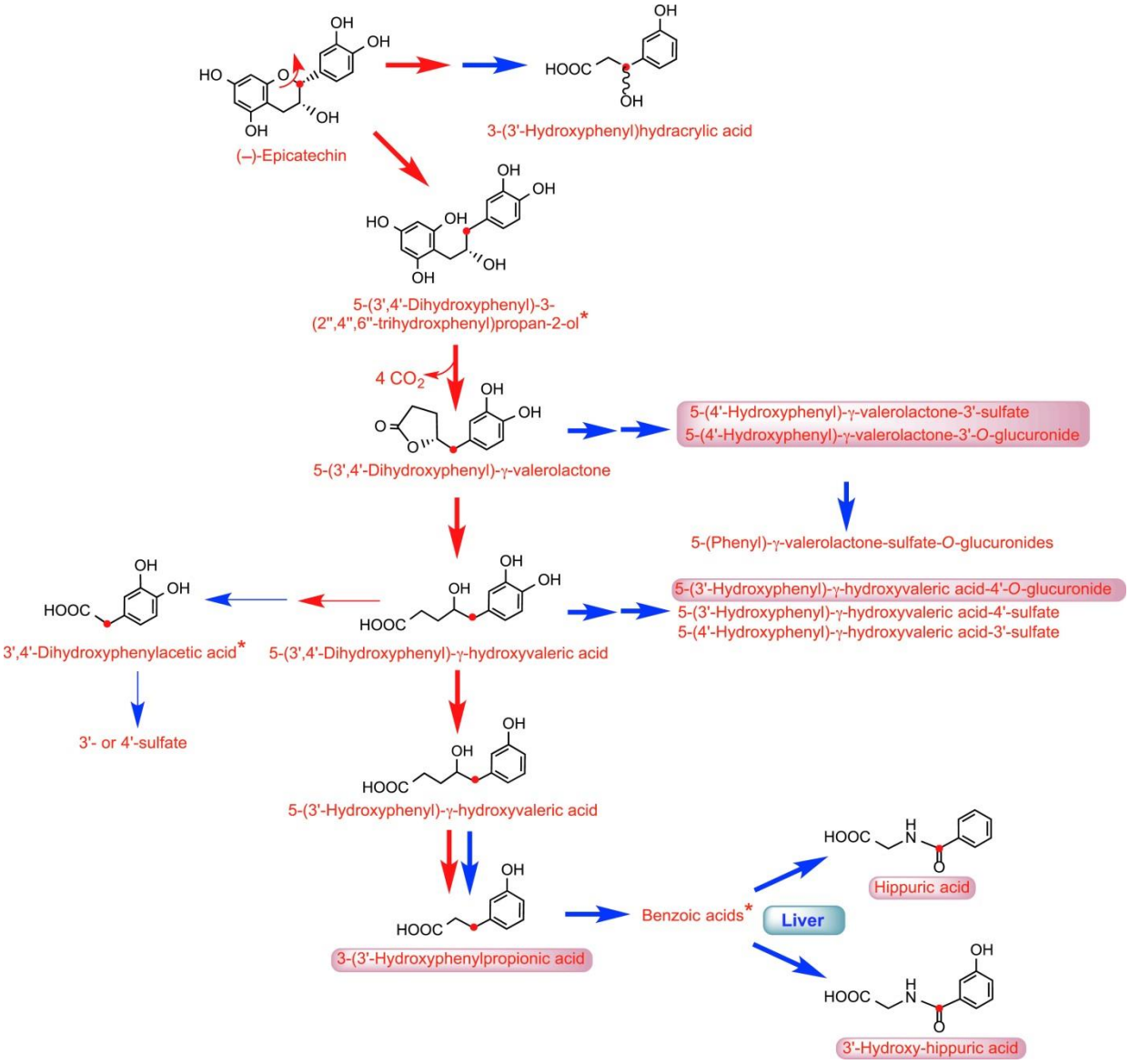


Figura 12. Rutas propuestas para el metabolismo por microbiota colónica de la (-) - epicatequina que pasa del intestino delgado al intestino grueso (flechas rojas) y posibles etapas catalizadas por enzimas de mamíferos en colonocitos y/o hepatocitos (flechas azules). Las flechas delgadas indican rutas menos importantes. Los nombres de metabolitos en los recuadros indican los principales productos se acumulan en el plasma después de la ingesta de (-) - epicatequina. Los asteriscos indican intermedios potenciales que no se acumulan en cantidades detectables (Borges et al., 2017).

Por otro lado, aunque varios estudios han observado que la procianidina B2 puede absorberse en el intestino delgado, la mayor proporción de este compuesto no se absorbe a nivel intestinal y pasa intacta al colon (>90%) (Urpi-Sarda et al., 2009; Del Río et al., 2013; Mena et al., 2014). Del mismo modo, se ha observado que las proantocianidinas oligoméricas y poliméricas no se degradan en las condiciones ácidas del estómago y no pueden absorberse a nivel del intestino delgado, por lo que pasan al intestino grueso sin ser alteradas (Del Río et al., 2013; Muñoz-Gónzalez et al., 2013). Una vez en el colon, las proantocianidinas son catabolizadas por la microbiota colónica, formando diversos ácidos fenólicos y compuestos aromáticos. Según estudios *in vitro*, se ha observado que la escisión del enlace entre los flavanoles no es una ruta principal (menos del 10 %), apareciendo como productos dominantes una serie de ácidos fenólicos con uno o dos hidroxilos fenólicos y de uno a cinco carbonos alifáticos en la cadena lateral (Stoupi et al., 2010abc). Por lo tanto, después de la ingesta de alimentos ricos en flavanoles, se forman varios ácidos fenólicos derivados, como el ácido 3-hidroxifenilpropiónico, el ácido 3-hidroxifenilacético y el ácido 3-hidroxibenzoico, así como las fenil-γ-valerolactonas (Spencer, 2003; Stockley, et al., 2012, Urpi-Sarda, et al., 2009; Martin et al., 2017).

En general, la (-)-epicatequina es altamente biodisponible, teniendo una tasa de absorción/excreción mayor que otros flavonoides, a excepción de las isoflavonas (Del Río et al., 2013). Además, se ha observado que las valerolactonas pueden ser un biomarcador potencial para futuros estudios de biodisponibilidad de los flavanoles (Ottaviani et al., 2016). Por el contrario, los diversos efectos beneficiosos para la salud atribuidos al consumo de proantocianidinas parece que no están relacionados directamente con la acción de estos compuestos, ya que no son absorbidos, y sí con la acción de sus metabolitos derivados del colon (Mena et al., 2014).

Adicionalmente, se ha observado que el metabolismo de los flavonoles, más concretamente, el perfil de metabolitos y catabolitos derivados de la epicatequina, era diferente en ratas, ratones y humanos, limitando la extrapolación de resultados interespecies (Ottaviani et al., 2016).

2.3.3. Biodisponibilidad de flavanonas

Las flavanonas son un grupo de flavonoides que se encuentran principalmente en las frutas del género *Citrus* (Abad-García et al., 2012). Las principales flavanonas en las naranjas son los 7-*O*-rutinósidos de hesperetina y naringenina, que se forman junto con los niveles más bajos de otros glucósidos, tales como hesperetina-7-*O*-rutinósido-3'-*O*-glucósido, hesperetina-7-*O*-glucósido, eriodictiol-7-*O*-rutinósido o 4'-*O*-metil-naringenina-7-*O*-rutinósido (Pereira-Caro et al., 2016).

En estudios de biodisponibilidad sobre el consumo de bebidas ricas en flavanonas, se han observado mayores niveles de glucurónidos de hesperetina en plasma a las 4 horas tras la ingesta, lo cual sugería que la absorción de estos compuestos fenólicos tenía lugar en el colon (Borges et al., 2010; Urpi-Sarda et al., 2012). Sin embargo, en un estudio posterior llevado a cabo por Borges et al. (2013), se ha observado la presencia de metabolitos de hesperetina en la orina de voluntarios ileostomizados. De esta forma, se ha estimado que en torno a una tercera parte de la hesperetina se absorbe en el tracto gastrointestinal superior, mientras que dos tercios se absorben en el intestino grueso (Borges et al., 2013).

En general, se ha observado que los derivados glucósidos de naringenina y hesperetina se absorben más rápidamente que sus homólogos rutinósidos (Nielsen et al., 2006; Bredsdorff et al., 2010). Así, las enzimas del intestino delgado (CBG y/o LPH) hidrolizan las flavanonas-*O*-glucósidos de forma más eficiente que las flavanona-*O*-rutinósidos, donde la acción de las enzimas es más limitada y, por tanto, la mayoría continúan hacia el intestino grueso, donde serán transformadas por la microbiota colónica (Kay et al., 2017).

Del mismo modo, Pereira-Caro et al., (2014) llevaron a cabo un estudio de biodisponibilidad de las flavanonas tras la ingesta de zumo de naranja, determinando la excreción urinaria de los metabolitos de hesperetina y narigenina, así como los ácidos fenólicos y aromáticos derivados. En este estudio, se determinó una absorción de en torno al 16 % de la ingesta de flavanona, siendo los principales metabolitos encontrados en orina los 3'-*O*- y 7-*O*-glucurónidos de hesperetina, hesperetina-3'-sulfato y los 4'-*O*- y 7-*O*-glucurónidos de naringenina. Del mismo modo, aunque la excreción de ocho catabolitos fenólicos aumentó significativamente tras la ingestión de zumo de naranja, el ácido 3-(3'-hidroxi-4'-metoxifenil)hidracrílico se seleccionó como posible marcador del consumo de naranja.

En la **Figura 13**, se puede observar una representación esquemática de los principales metabolitos y catabolitos de hesperetina y naringenina excretados en orina en función del tiempo tras la ingesta de zumo de naranja en humanos. Durante las primeras 5 horas, los metabolitos mayoritarios derivados de hesperetina fueron la hesperetina-3-*O*-glucurónido y

hesperetina-3-*O*-sulfato, mientras que naringenina-4-*O*-glucurónido y naringenina-7-*O*-sulfato fueron los principales derivados de la naringenina (**Figura 13**). Entre las 5 y 24 horas, se observan una gran cantidad de catabolitos derivados de la hesperetina, tales como ácido dihidroferulico-3'-sulfato, ácido dihidroferulico-3'-*O*-glucurónido, ácido 3-(3'-hidroxi-4'-metoxifenil)hidracrílico, ácido dihidroferúlico y ácido 3,4-dihidroxifenilacético. El ácido 4-hidroxifenilacético fue el principal catabolito derivado de la naringenina (**Figura 13**). Del mismo modo, varios catabolitos fueron derivados comunes de ambas flavanonas, como el ácido 3-(fenil)propionico, el ácido benzoico-4-sulfato, el ácido 4-hidroxihipurico y el floroglucinol (**Figura 13**).

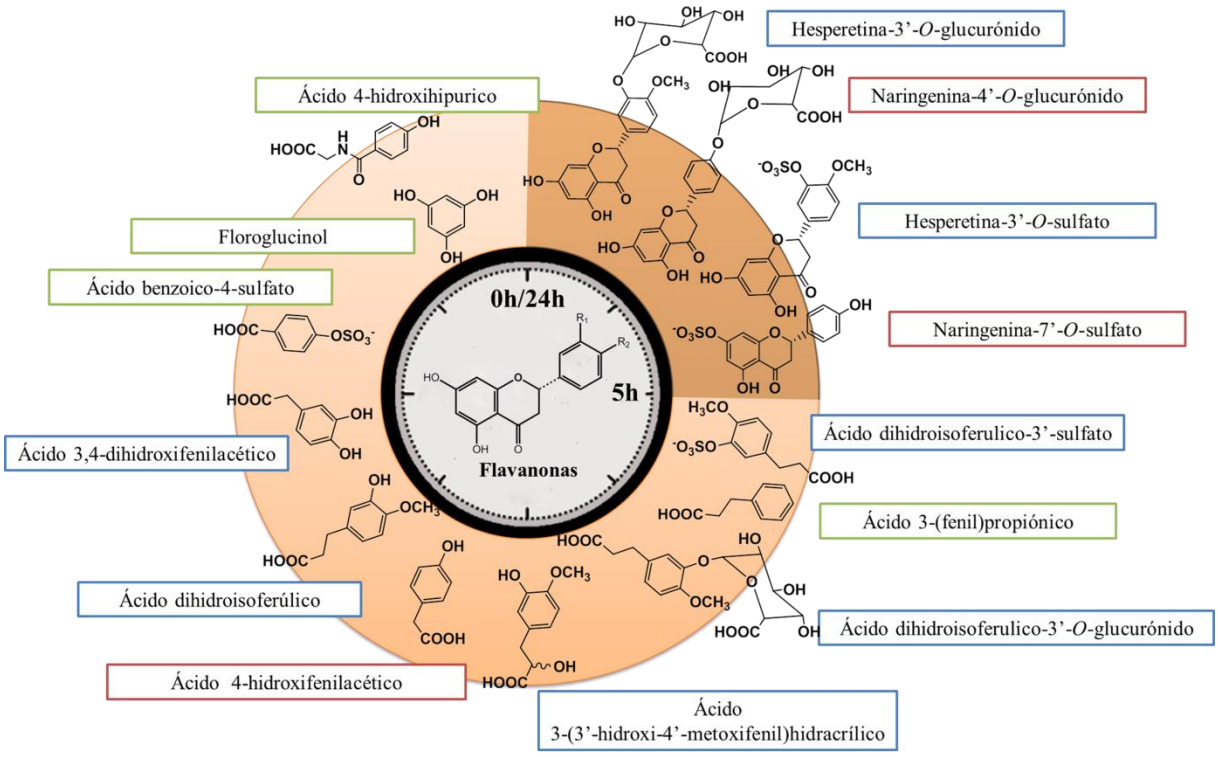


Figura 13. Representación esquemática del metabolismo de las flavanonas en la orina en humanos en función del tiempo. Recuadro azul: derivados de la hesperetina. Recuadro rojo: derivados de la naringenina. Recuadro verde: derivados de hesperetina o naringenina.

En la **Figura 14**, se observa la ruta catabólica propuesta para la naringenina y el ácido ferúlico mediada por la acción de las enzimas presentes en el colon. Del mismo modo, la **Figura 15** muestra la ruta catabólica propuesta para la hesperetina por la acción de las bacterias presentes en el colon.

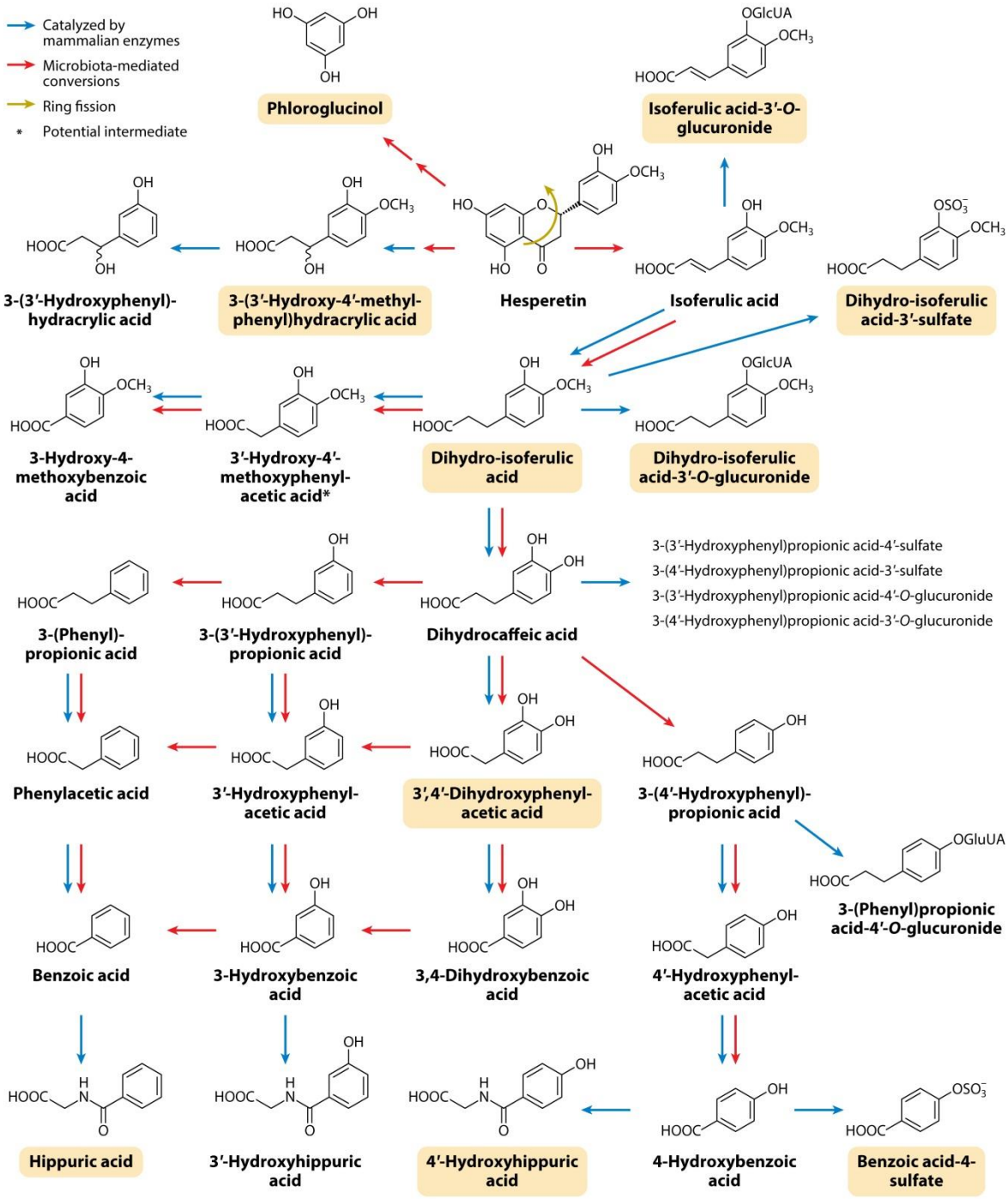


Figura 14. Rutas propuestas para el metabolismo colónico de la hesperetina liberada por la escisión de hesperetina-7-*O*-rutinósido tras el consumo de zumo de naranja. Los recuadros incluyen los compuestos principales en la orina después de la ingesta (24 horas). La flecha amarilla indica escisión de anillo. Las flechas rojas son conversiones mediadas por microbiota y las flechas azules indican pasos catalizados por enzimas de mamíferos. Los asteriscos indican productos intermedios potenciales que no se acumularon en cantidades detectables. Abreviatura: GlcUA, ácido glucurónico (Kay et al., 2017).

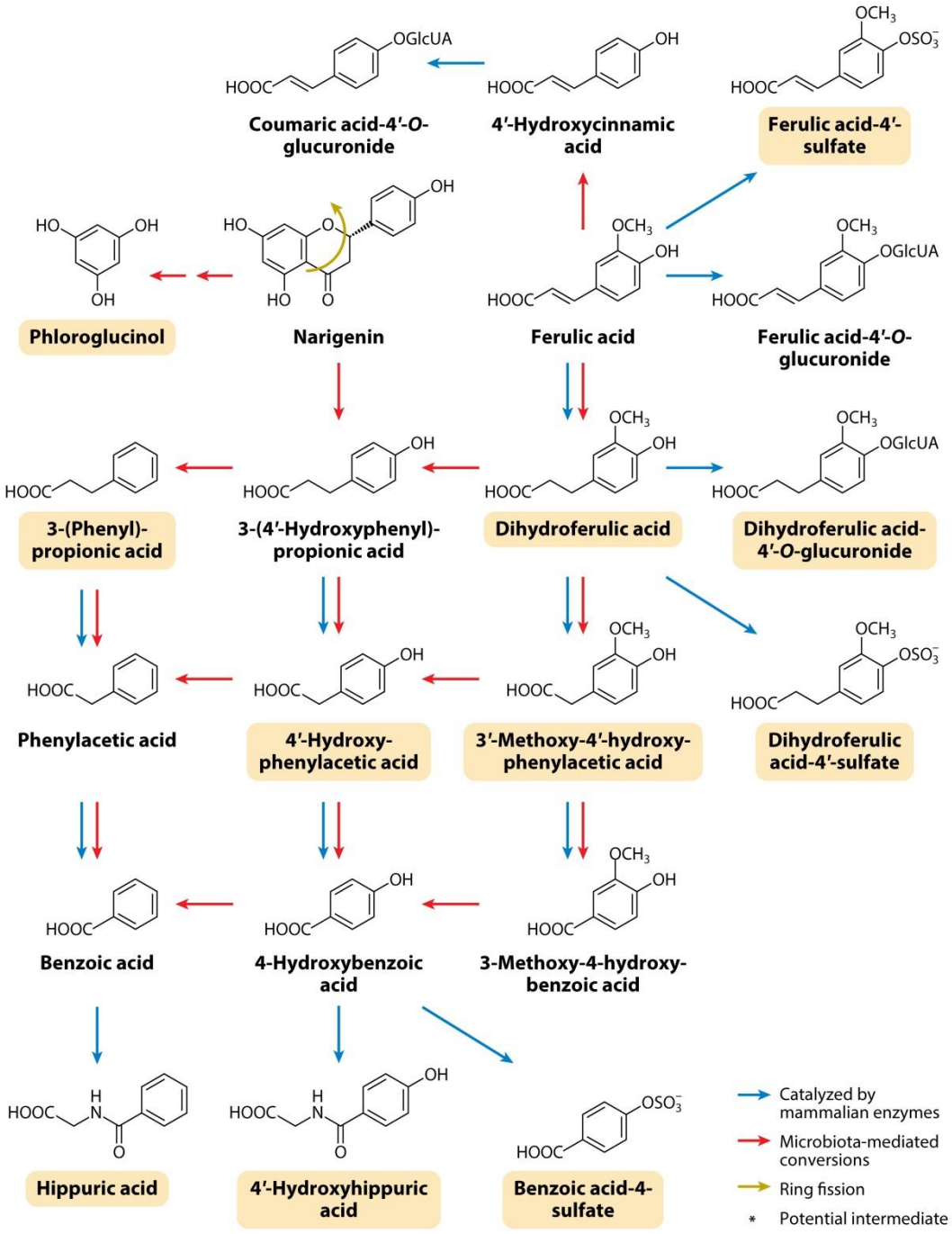


Figura 15. Rutas propuestas para el metabolismo de naringenina y ácido ferúlico liberado por escisión de naringenina-7-*O*-rutinósido y ácido ferúlico-4'-*O*-glucósido tras el consumo de zumo de naranja. Los recuadros incluyen los compuestos principales en la orina después de la ingesta (24 horas). La flecha amarilla indica escisión de anillo. Las flechas rojas son conversiones mediadas por microbiota y las flechas azules indican pasos catalizados por enzimas de mamíferos. Los asteriscos indican productos intermedios potenciales que no se acumularon en cantidades detectables. Abreviatura: GlcUA, ácido glucurónico (Kay et al., 2017).

2.4. Técnicas analíticas para la determinación de Compuestos Fenólicos y Derivados

En los últimos años, la cromatografía ha sido la técnica más utilizada para determinar compuestos fenólicos y derivados en muestras de orina; especialmente, la cromatografía líquida de alta eficacia (HPLC) y la cromatografía de gases (GC). Del mismo modo, el espectrómetro de masas (MS) ha sido el detector más usado, ya que proporciona información estructural específica y tiene una alta capacidad de detección (Pereira-Caro et al., 2016; Feliciano et al., 2016). Estas técnicas permiten obtener un perfil completo de los compuestos fenólicos y derivados en muestras biológicas, ofreciendo una información valiosa sobre la biodisponibilidad de dichos compuestos (Rodríguez-Mateos et al., 2014).

En la **Tabla 5**, se resumen algunos estudios centrados en la identificación y cuantificación de compuestos fenólicos y derivados en muestras biológicas; principalmente, en trabajos publicados a partir de 2010 y hasta la actualidad. Estos estudios pueden clasificarse en modelos *in vitro* y modelos *in vivo*, tanto en animales como en humanos.

En los últimos años, numerosos estudios han empleado modelos de incubación fecal *in vitro* usando compuestos puros o alimentos y analizando los productos de la transformación de los compuestos fenólicos mediante técnicas cromatográficas. Estos estudios intentan dilucidar las posibles rutas catabólicas de los compuestos fenólicos mediadas por la microbiota e identificar sus potenciales productos intermedios. Así, por ejemplo, se han incubado distintos productos, como café o zumo de naranja, con muestras fecales, permitiendo establecer rutas potenciales de degradación de compuestos fenólicos (Ludwig et al., 2013; Pereira-Caro et al., 2015) (**Tabla 5**).

En general, la mayoría de las investigaciones sobre biodisponibilidad se han realizado en modelos animales y/o en voluntarios tras el consumo de un solo producto. Estos estudios permiten el análisis de los metabolitos en muestras biológicas, siendo la orina el tipo de muestra más empleada, seguida de las muestras de plasma y heces (**Tabla 5**). Por un lado, la farmacocinética de los metabolitos en el plasma proporciona una información útil, aunque las estimaciones de valores como el área bajo la curva no son esencialmente datos cuantitativos y precisos sobre la absorción. De este modo, la excreción urinaria proporcionaría unos valores más realistas, teniendo en cuenta la posibilidad de secuestro en los tejidos, que parece producirse en niveles bajos (Del Rio et al., 2013). Por otro lado, el empleo de modelos animales permite la determinación de metabolitos en órganos diana, lo cual no es posible en estudios clínicos en humanos, limitando la extrapolación de resultados interespecies (Ottaviani et al., 2016).

Tabla 5. Determinación de compuestos fenólicos por métodos cromatográficos.

Derivados de Compuestos fenólicos	N	Muestras	Producto consumido	Extracción	Detección	T (min)	Parámetros de validación			Referencias
							L	P	R	
In vitro										
Ác. clorogénicos	11	Fecal	Café	SPE (SDB-L) *(MSTFA)*	GC-MS / HPLC-PDA-MS^n	50	SI	X	X	Ludwig et al., 2013
Flavanonas	6	Fecal	Zumo de naranja	SPE (SDB-L) *(MSTFA)*	GC-MS / HPLC-PDA-MS^n	50	X / SI	X	X	Pereira-Caro et al., 2015
Roedores										
Flavanoles Antocianidinas	16	Órganos	Hollejo de uva	LSE-µSPE (HLB)	UPLC–MS^2	12,5	SI	X	SI	Serra et al., 2011
Ác. clorogénicos	11	Orina, Plasma, hígado	Ácido 5-cafeoilquínico	β-Gl/Ss, SPE (HLB)	UPLC–MS	12,5	SI	SI	SI	Oliveira et al., 2013
Flavanoles	23	Orina, Plasma, Órganos	Cianidina-3-glucósido	SPE (HPD)	UPLC–MS^2	17	SI	SI	SI	Gasperotti et al., 2014
Flavanoles Ác. fenólicos	30	Plasma	Extracto de proantocianidinas de semillas de uvas	µSPE (HLB)	HPLC-ESI-TOF/MS HPLC-ESI-MS^2	16	SI	SI	SI	Margalef et al., 2014
Flavanoles	38	Colon	Vitaflavan ® (Extracto de semillas de uvas)	LLE, SPE (HLB)	UPLC-MS^2	10	SI	SI	SI	Goodrich et al., 2014
Proantocianidinas	53	Orina, Plasma, Órganos	Procianidina B2	MeOH	UPLC-DAD-ESI-IT-TOF-MS^n	47	X	X	X	Xiao et al., 2017
Humanos										
Flavanoles	15	Orina, Fecal	Té verde	SPE (SDB-L) *(N,O-BSTFA+1%TMCS)*	GC-MS	50	X	X	SI	Roowi et al., 2010
Flavanoles	39	Orina, Plasma	Té verde	SPE (HLB)	HPLC-MS^2	15	X	X	X	Del Rio et al., 2010
Ác.hidroxicinámicos	50	Plasma	Café	ACN	HPLC-MS	45	SI	X	SI	Redeuil et al., 2011
Flavonoides Ác. fenólicos	47	Fecal	Cápsulas de flavanoles	ACN/H_2O	UPLC-ESI-TQMS	18	SI	SI	SI	Sánchez-Patán et al., 2011
Flavanoles	138	Orina	Té verde y negro	SPE (HLB)	HPLC-FTMS	85	X	X	SI	van der Hooft et al., 2012
Flavanoles	15	Orina, plasma	Chocolate	ACN	HPLC-MS^2	8	SI	X	SI	Actis-Goretta et al., 2012
Comp. fenólicos	43	Orina	Compuestos fenólicos	ACN, β-Gl/Ss	UHPLC-MS^2	10	SI	SI	SI	Magiera et al., 2012

Flavonoides Ác. fenólicos	32	Orina	Jarabe de arándanos	SPE (Evolute ABN), SPE (Isolute ENV+)	UPLC-ESI-QTOF-MS	35	X	X	X	Iswaldi et al., 2013
Flavanoles Ác. fenólicos	61	Orina	Vino tinto desalcoholizado	β-Gl/Ss; µSPE (MCX o HLB)	UPLC−MS^2	3,5	X	X	X	Boto-Ordóñez et al., 2013
Antocianidinas	25	Orina, Plasma, Fecal	$^{13}C_5$-cianidina-3-glucosido	SPE (Strata-X)	HPLC-MS^2	32,5	X	X	SI	Czank et al., 2013
Flavonoides Ác. fenólicos	35 25	Fecal	Vino tinto	ACN SPE (HLB)	UPLC-ESI-MS^2	18	SI	SI	SI	Muñoz-Gónzalez et al., 2013 Muñoz-Gónzalez et al., 2014
Antocianidinas	45	Orina[A], Plasma[B], Fecal[C]	Antocianidinas	SPE (DSC-18[A]; Strata-X[B]; Bond Elute C18[C])	HPLC-MS^2	30	SI	SI	SI	de Ferrars et al., 2014
Antocianidinas Elagitaninos	38	Orina, Plasma	Frambuesa roja	ACN	HPLC-PDA-MS^2	22	X	X	X	Ludwig et al., 2015
Flavonoides Ác. fenólicos	67	Orina, Plasma	Zumo de arándanos	µSPE (HLB)	UHPLC-QTOF-MS	10	SI	SI	SI	Feliciano et al., 2016
Flavanonas Ác. fenólicos	84 48	Orina Plasma	Zumo de naranja	Filtrada ACN	HPLC-HRMS	45	X	X	X SI	Pereira-Caro et al., 2016
Flavonoides Ác.hidroxicinámicos	9	Orina, Plasma	Extracto de bayas de aronia	SPE (C18)	UHPLC-MS	25	SI	SI	SI	Xie et al., 2016
Antocianidinas Flavanoles Ác. elágico y gálico	40	Orina Plasma Fecal	Madroño	µSPE (HLB) Filtros FTA® DMPK card LSE	UHPLC-MS^2	30	SI	SI	SI	Mosele et al., 2016
Flavanoles	23	Orina, Plasma	[2-^{14}C](-)-epicatequina	SPE (HLB)	HPLC-MS^2-DR	40	SI	X	X	Ottaviani et al., 2016
Flavanoles	10	Orina	Té verde	Filtrada	UHPLC-MS/MS	9	SI	SI	SI	Brindani et al., 2017
Ac. fenólicos Flavonoides Estilbenos	77	Orina	Hollejo de uva	0,1 M HCl	HPLC-ESI-LTQ-Orbitrap-MS	15	X	X	X	Sasot et al., 2017
Flavonoides Dihidrochalcona	6	Orina	Manzana	β-Gl/Ss, ACN	HPLC-MS^2	9	SI	SI	SI	Saenger et al., 2017
Flavonoles	45	Orina, Plasma	Yerba mate	LLE	HPLC-ESI-QTOF	20	X	X	X	Gómez-Juaristi et al., 2018

N: número de analitos. t: tiempo. L: Linearidad y límites. P: Precisión. R: Recuperación. SPE: Extracción en fase sólida. LSE: Extracción sólida-líquida. LLE: Extracción líquida-líquida. β-Gl/Ss: Hidrólisis con β-glucuronidasas/sulfatasas. MeOH: Metanol. ACN: Acetonitrilo. HCL: Ácido hipoclorito. GC: cromatografía de gases. HPLC/UHPLC: cromatografía de líquidos de alta resolución. PDA: Detector de matriz de fotodiodos. MS^x: Espectrómetro de masas. IT: Trampa iónica. DR: Detector de radioactividad. MSTFA: *N*-metil-*N*-(trimetilsilil) trifluoroacetamida. BSTFA: *N,O*-bis (trimetilsilil) trifluoroacetamida. TMCS: trimetilclorosilano. X: no determinado.

Es interesante destacar que la cuantificación de los metabolitos es compleja, debido a que los estándares de referencia no suelen estar disponibles comercialmente y son muy complicados y costosos de sintetizar (Barron et al., 2012; Zhang et al., 2013b). De esta forma, la mayoría de los análisis son solo cualitativos o semicuantitativos, debido a varios factores, como la composición compleja de muestras biológicas, las posibles formas regioisoméricas y la baja ionización de algunos derivados que pueden obstaculizar la identificación y la cuantificación absoluta de estos metabolitos (Brindani et al., 2017). En numerosos estudios, las muestras se tratan con enzimas hidrolíticas, principalmente, glucuronidasas y sulfatasas; y las agliconas liberadas se cuantifican por LC usando detectores de absorbancia, fluorescencia o electroquímicos (Lee et al., 2002; Chow et al., 2005; Henning et al., 2005). Sin embargo, este tipo de análisis presenta numerosas desventajas, ya que la actividad y eficacia de las enzimas que se usan son muy variables y, sobre todo, subestima la biodisponibilidad de los compuestos fenólicos y no proporciona información sobre los metabolitos conjugados específicos (Donovan et al., 2006; Saha et al., 2012). Adicionalmente, aunque se han empleado enfoques semicuantitativos usando agliconas, la falta de estándares dificulta la calibración precisa y la cuantificación (Nagy et al., 2011). De este modo, en los últimos años, la síntesis química de estos compuestos y el uso de cromatografía acoplada a espectrometría de masas, sin la necesidad de usar la hidrólisis enzimática, ofrecen una gran ventaja para el desarrollo de los métodos analíticos para el estudio del metabolismo y la biodisponibilidad de compuestos fenólicos (Brindani et al., 2017).

Por otro lado, otro factor a tener en cuenta durante el análisis de metabolitos en muestras biológicas es la preparación de la muestra previamente al análisis por cromatografía. En la mayoría de los estudios, las muestras biológicas de orina, plasma y heces se tratan para extraer y concentrar los analitos de interés y disminuir el efecto de la matriz, mejorando los límites de detección y evitando interferencias. Los métodos de extracción más empleados son la extracción líquida con solventes orgánicos, como metanol o acetonitrilo, y la extracción en fase sólida (SPE). Los cartuchos de SPE más usados son SDB-L para los métodos de GC y HLB para los métodos de HPLC (**Tabla 5**). Los cartuchos SDB-L están formados por un copolímero estireno-divinilbenceno, proporcionando una selectividad a compuestos hidrofóbicos. Por otro lado, los cartuchos de HLB están formados por un sorbente equilibrado hidrofílico-lipofílico, en concreto, un copolímero divinilbenceno y *N*-vinilpirrolidona. Así, la *N*-vinilpirrolidona tiene la capacidad de retener los analitos polares, mientras que el divinilbenceno retiene los más hidrofóbicos (Savage et al., 2011). Se ha observado que los cartuchos HLB tienen una capacidad de recuperación superior al 85 % para una gran cantidad de metabolitos derivados de compuestos fenólicos (van der Hooft et al., 2012; Mosele et al., 2016; Feliciano et al., 2016). Por el contrario, estos cartuchos no tienen buena recuperación para los flavanoles, especialmente, las procianidinas (Muñoz-González et al., 2013, 2014).

En el caso de los métodos GC, es necesario un paso de derivatización para aumentar las propiedades volátiles de los compuestos fenólicos. Así, los reactivos de derivatización más usados son *N*,*O*-bis (trimetilsilil) trifluoroacetamida (BSTFA) o *N*-metil-*N*-(trimetilsilil) trifluoroacetamida (MSTFA) (Nolvachai & Marriott, 2013). En un estudio comparativo entre BSTFA con 1 % de trimetilclorosilano y MSTFA, se observó una mejor intensidad de señal para compuestos fenólicos y metabolitos con el segundo reactivo (Magiera et al., 2011). Además, los derivados de MSTFA son más estables en comparación con los derivados de BSTFA durante el almacenamiento (Chan et al., 2011).

En general, el tiempo de análisis en los métodos de cromatografía líquida es inferior a media hora, mientras que en cromatografía de gases, está alrededor de los 50 minutos. Teniendo en cuenta la validación de métodos, se observa que en torno a la mitad de los trabajos evaluados en la **Tabla 5** han sido validados. Así, gran parte de estos estudios no ofrecen información acerca de la sensibilidad, precisión o recuperación del método empleado, siendo estos unos parámetros importantes para demostrar la adecuación de los métodos y la obtención de datos de calidad.

3. Validación de métodos analíticos

La validación de un método analítico tiene como objetivo principal garantizar que la medida de los analitos durante un análisis se acerque lo suficiente al valor real presente en la muestra (Gónzalez & Herrador, 2007). De esta forma, la validación es necesaria para confirmar la idoneidad del método aplicado sobre un tipo de material específico, así como a un rango de concentración definida del analito (Tavernier et al., 2004).

En general, antes de iniciar una validación, hay que definir el alcance del método, integrando tanto el sistema analítico como los requisitos analíticos. De esta forma, el sistema analítico comprende el propósito y el tipo de método, el tipo y el rango de concentración de los analitos, los tipos de matrices donde se aplica y un protocolo del método. Por otro lado, es necesario establecer los requisitos analíticos, donde se reflejarán los criterios mínimos de idoneidad y los diferentes criterios de funcionamiento del método. Los criterios establecidos formarán la base de la aceptabilidad final de los datos analíticos y del método validado (Green, 1996; Tavernier et al., 2004).

Existen diversas guías elaboradas por diferentes organizaciones y autores que recogen las pautas o directrices del proceso de validación de un método analítico. Estas pautas pueden variar ligeramente entre las guías, aunque su finalidad es común. Las instituciones que han elaborado guías oficiales para la validación de métodos que constituyen una referencia son ICH (*Internacional Conference for Harmonization*), EURACHEM, IUPAC (*Internacional Union of Pure and Apply Chemistry*), FDA (*Food and Drug Administration*), LGC (*Laboratory of the Government Chemistry*), ISO (*International Organization for Standardization)*, AOAC (*Association of Analytical Communities)* (ICH, 1995, 1996; EURACHEM, 1998, 2014; IUPAC 1998, 2002; FDA 2001; LGC, 2003; ISO, 2005; AOAC, 2007) o el libro de validación de métodos elaborado por Huber (2010).

El grado y la elección de los parámetros de validación a evaluar dependen de la aplicación previa del método analítico. Cuando un método se ha validado previamente de acuerdo con un protocolo internacional, el laboratorio no necesita realizar extensos estudios internos de validación. De esta forma, solo se debe verificar que puede lograr las mismas características de rendimiento que las delineadas en el estudio colaborativo. Del mismo modo, cuando se trata de un método completamente validado, pero aplicado a una nueva matriz, solo se requiere una validación limitada. Por el contrario, cuando se desarrollan nuevos métodos, sí es necesaria una validación más amplia (Tavernier et al., 2004).

Por todo ello, el propósito de un método analítico es obtener un resultado cualitativo y/o cuantitativo con un nivel de incertidumbre aceptable. En la práctica, la validación del método se

realiza mediante la evaluación de una serie de parámetros, como linealidad o rango operativo, aplicabilidad, selectividad, sensibilidad [límite de detección (LDD) y límite de cuantificación (LDC)], precisión y recuperación, entre otros.

3.1. Parámetros para la validación

3.1.1. Selectividad y Especificidad

La selectividad es la capacidad de un método analítico para diferenciar y cuantificar el analito en presencia de otros componentes en la muestra. Para la selectividad, es necesario analizar muestras sin el analito de interés (blanco) en la matriz biológica apropiada, realizando pruebas de interferencia y garantizando su selectividad en el límite inferior de la cuantificación. Del mismo modo, si el método está destinado a cuantificar más de un analito, debe probarse cada uno de ellos para garantizar que no hay interferencias (FDA, 2001). En general, varias instituciones consideran la selectividad y la especificidad como un mismo parámetro (EURACHEM, 1998; IUPAC, 2002; AOAC, 2007). Para las técnicas cromatográficas, la especificidad se puede evaluar comparando el tiempo de retención de estándares con los analitos en la matriz biológica o, en el caso de la espectrometría de masas, calculando el error de masa (diferencia entre la m/z prevista y la m/z observada).

3.1.2. Linealidad

La linealidad puede definirse como la capacidad de un método analítico, dentro de un intervalo específico, para obtener resultados que sean directamente proporcionales a la concentración del analito en la muestra (ICH, 1995). La linealidad se determina analizando un mínimo de 6 medidas a distintas concentraciones de los estándares en un rango de concentración esperado y elaborando una curva de calibración para cada analito en la muestra (FDA, 2001).

3.1.3. Sensibilidad: Límites de detección (LDD) y Límites de cuantificación (LDC)

El límite de detección (LDD) es la mínima concentración o cantidad de analito presente en la muestra y que puede ser detectada, aunque no necesariamente cuantificada, bajo las condiciones experimentales descritas. Por otra parte, el límite de cuantificación (LDC) se entiende como la mínima concentración o cantidad de analito en la muestra que es posible cuantificar (ICH, 1995). Por lo tanto, el LDC es un valor cuantitativo, mientras que el LDD es un valor cualitativo. Existen diferentes procedimientos para calcular los LDD y LDC (ICH, 1996):

1. *Evaluación visual*: Se utiliza en métodos de análisis no instrumentales sin una señal numérica. Los límites se pueden determinar a partir del análisis de muestras con concentraciones conocidas y decrecientes de analito, estableciéndose visualmente la mínima concentración detectable (LDD) o cuantificable (LDC).

2. *Basados en la señal/ruido*: La determinación de la señal ruido (señal residual con concentración cero del analito) se lleva a cabo comparando la medida de la señal de muestras con bajas concentraciones conocidas del analito con la medida de la señal del blanco. Así, el LDD será la concentración de analito que proporcione una señal tres veces superior al ruido de fondo, mientras que el LDC será igual a la concentración de analito que proporcione una relación señal/ruido de 10:1.

3. *Basados en las desviaciones estándar de la respuesta y la pendiente*: Consiste en el estudio de la recta de calibrado del analito a concentraciones bajas. De esta forma, los límites se calculan como la relación entre la desviación estándar de la ordenada en el origen multiplicado por 3,3 (LDD) o 10 (LDC) respecto a la pendiente de la resta.

3.1.4. Precisión

La precisión puede definirse como el grado de concordancia entre los resultados de ensayos independientes, obtenidos en unas condiciones bien definidas (ICH, 1995). El objetivo principal del estudio de la precisión es conocer la variabilidad o errores del método analítico, expresados como porcentaje de desviación estándar relativa (RSD %). Los factores que influyen sobre los resultados de un ensayo no pueden controlarse siempre (analista, equipo instrumental, reactivos, tiempo, etc.), por lo que el estudio de la precisión es importante.

La precisión puede evaluarse a tres niveles: repetibilidad o precisión intradía, precisión intermedia o interdía y reproducibilidad (Huber, 2010).

1. La repetibilidad o precisión intradía es la medida del grado de dispersión de una serie de medidas realizadas con los mismos equipos y reactivos, en un espacio de tiempo corto (en una misma sesión de trabajo) y por el mismo operador.

2. La precisión intermedia o interdía es la medida de la dispersión de los resultados obtenidos por un método analítico en un espacio de tiempo más amplio que la repetitividad, modificando ciertas condiciones de operación: diferentes días, analistas, equipos, etc.

3. La reproducibilidad expresa la precisión entre laboratorios, formando parte de los estudios interlaboratorio.

3.1.5. Exactitud

La exactitud de un método analítico se describe como el grado de concordancia entre el valor experimental obtenido y el valor aceptado como referencia, por ejemplo, en los resultados de un método normalizado o materiales de referencia certificados (ICH, 1995; FDA, 2001). La

exactitud se determina mediante análisis repetidos de muestras que contienen cantidades conocidas del analito, con un mínimo de cinco determinaciones.

3.1.6. Recuperación

El grado de recuperación puede definirse como la respuesta del detector obtenida a partir de una cantidad del analito añadido y recuperado de una matriz biológica, comparado con la respuesta del detector obtenida con la concentración del estándar puro. La recuperación se expresa en porcentaje y depende de la concentración del analito o la matriz y del procesado de la muestra (FDA, 2001). La recuperación puede usarse como alternativa a la exactitud cuando no se dispone de materiales de referencia certificados (Huber, 2010).

3.1.7. Efecto matriz

El efecto matriz se puede definir como el efecto de todos los componentes de la muestra distintos al analito de interés. El efecto matriz para cada analito se puede evaluar comparando la pendiente de la curva de calibración preparada en la matriz biológica a analizar con la extracción y la curva de calibración preparada en un solvente (Gónzalez & Herrador, 2007). La falta de diferencias significativas entre las curvas muestra que no existe efecto matriz (Tavernier et al., 2004). La disminución del efecto matriz puede llevarse a cabo mediante métodos de extracción, como el SPE (Gasperotti et al., 2014).

JUSTIFICACIÓN, HIPÓTESIS Y OBJETIVOS

Justificación, Hipótesis y Objetivos

Justificación

La Seguridad Alimentaria tiene como principal finalidad la protección de la salud de las personas en relación con los alimentos ingeridos. A principios del presente siglo, tras las crisis alimentarias acontecidas en Europa a finales del siglo XX, se definieron los principales pilares en los que se basa la seguridad alimentaria actual evaluación, gestión y comunicación del riesgo. De esta forma, se creó la Autoridad Europea de Seguridad Alimentaria (EFSA), un organismo encargado de facilitar la evaluación del riesgo, que debe basarse en el mejor conocimiento científico disponible. En este contexto, esta institución elaboró en 2011 una opinión científica sobre la formación de aminas biógenas en alimentos fermentados, en la que se pone de manifiesto la importancia del control de estos compuestos causantes de reacciones adversas en los consumidores. Así, es importante el seguimiento de las aminas biógenas en los alimentos fermentados durante el proceso de producción así como a lo largo de la cadena alimentaria para poder establecer sistemas de control y reducir los riesgos de intoxicación. Para realizar una mejor evaluación del riesgo, la EFSA señala que es preciso disponer de mayor conocimiento científico en relación a la presencia de aminas biógenas en alimentos fermentados, especialmente respecto a su toxicidad, controles en la producción, higiene de los procesos, criterios de inocuidad de los alimentos y la validación de los métodos de análisis (EFSA, 2011), aspecto este último que se aborda en esta Tesis.

Por otra parte, la EFSA también es la encargada de evaluar las Declaraciones de Propiedades Saludables de los alimentos (Reglamento 1924/2006). En los últimos años, se han propuesto en torno a 30 solicitudes de declaraciones saludables relacionadas con los compuestos fenólicos en varios alimentos, de las cuales sólo dos han sido autorizadas: el hidroxitirosol en aceite de oliva virgen (EFSA 2011b) y los flavanoles en cacao) (EFSA 2012). En consecuencia, el Panel de Productos Dietéticos, Nutrición y Alergias (NDA) perteneciente a la EFSA ha elaborado una guía de orientación para la preparación y presentación de solicitudes de autorización de declaraciones saludables, comprendida en los artículos 13(5), 14 y 19 del Reglamento (CE) nº 1924/2006. Así, para demostrar una declaración saludable hay que caracterizar el alimento, definir su actividad fisiológica, así como su disponibilidad y plausibilidad biológica. Por todo ello, para poder evaluar las funciones biológicas de los compuestos fenólicos es imprescindible conocer su biodisponibilidad, término que engloba la absorción, distribución, metabolismo y excreción (ADME) (Kay et al., 2016). Además, es importante evaluar e identificar las transformaciones de estos compuestos a lo largo del tracto gastrointestinal, ya que se ha observado que las propiedades bioactivas de los metabolitos y catabolitos microbianos, aun

siendo los principales implicados en los efectos beneficiosos, suelen ser diferentes a la de sus compuestos originales presentes en los alimentos (Duda-Chodak et al., 2015).

En general, las dos clases de compuestos químicos, tanto las aminas biógenas como los compuestos fenólicos, suelen estar presentes en bajas concentraciones en las muestras de alimentos y biológicas. Además, debido a la gran complejidad de este tipo de muestras, es necesario desarrollo de técnicas analíticas lo suficientemente sensibles, fiables y robustas para la determinación de estos compuestos en diferentes matrices.

Según lo expuesto anteriormente, la **hipótesis** básica de la Tesis supone que el desarrollo y validación de métodos analíticos va a permitir obtener datos de calidad y mejores resultados de fiabilidad, sensibilidad, resolución, selectividad, precisión y recuperación para la determinación de aminas biógenas en diferentes matrices alimentarias así como la determinación de metabolitos y catabolitos derivados de la ingesta de alimentos ricos compuestos fenólicos en muestras biológicas.

La presente Tesis Doctoral tiene como **objetivo general** poner a punto y validar métodos analíticos específicos basados en técnicas cromatográficas para caracterizar y cuantificar compuestos con actividad biológica, como son las aminas biógenas, compuestos fenólicos y sus derivados, en productos vegetales, bebidas fermentadas y muestras biológicas obtenidas de estudios de biodisponibilidad.

Para la consecución de este objetivo general se han propuesto los siguientes objetivos parciales:

- **Objetivo 1:** Realizar una revisión bibliográfica sobre el estado del arte de la determinación de aminas biógenas en bebidas fermentadas, centrándose principalmente en la novedad, mejora y optimización de los métodos analíticos desde 2010 hasta el presente.

- **Objetivo 2:** Evaluar el perfil de aminas biógenas, aminoácidos y amonio en puré de fresa y productos obtenidos tras su fermentación glucónica.

  - Objetivo específico 2.1: Adaptar un método analítico para la determinación de aminas biógenas, aminoácidos y amonio mediante cromatografía de líquidos acoplada a detector de fotodiodos y usando DEEMM como reactivo derivatizante.

  - Objetivo específico 2.2: Comparar respecto al perfil de aminas biógenas, aminoácidos y amonio, diferentes procesos de fermentación glucónica llevados a cabo mediante cultivo superficial por distintas bacterias acéticas (*Acetobacter malorum,Gluconobacter oxydans y Gluconobacter japonicus)* y seleccionar la especie más adecuada para elaboración de estos fermentados de fresa.

- Objetivo específico 2.3: Monitorizar el perfil de aminas biógenas, aminoácidos y amonio de varias fermentaciones glucónicas de puré de fresa llevadas a cabo mediante cultivo sumergido por la bacteria acética seleccionada.

- **Objetivo 3:** Evaluar el perfil de aminas biógenas en productos de fermentación alcohólica y acética.

- Objetivo específico 3.1: Poner a punto y validar un método analítico para la determinación de aminas biógenas y en diferentes matrices mediante cromatografía de líquidos acoplada a detector de fluorescencia empleando AQC como reactivo derivatizante.

- Objetivo específico 3.2: Determinar las concentraciones de aminas biógenas en vinagres comerciales (vinagre de vino tinto, blanco, Jerez, Pedro Ximénez, manzana y balsámico).

- Objetivo específico 3.3: Controlar la evolución de aminas biógenas en botellas abiertas de diferentes vinos durante varios días mantenidas a diferentes condiciones de conservación (temperatura y vacío).

- **Objetivo 4:** Validar métodos de análisis para determinar los compuestos fenólicos y sus metabolitos en diferentes matrices biológicas (orina, plasma y heces).

- Objetivo específico 4.1: Validar un método analítico para la determinación de compuestos fenólicos y sus metabolitos en diferentes matrices biológicas mediante cromatografía de líquidos acoplado a un detector de espectrometría de masas de alta resolución.

- Objetivo específico 4.2: Identificar y cuantificar los compuestos fenólicos y sus metabolitos en diferentes matrices biológicas tras la ingesta de un extracto de vino tinto enriquecido en procianidinas por ratas.

- **Objetivo 5:** Validar y comparar dos técnicas analíticas para la determinación de compuestos fenólicos en orina tras el consumo de zumo de naranja.

- Objetivo específico 5.1: Validar un método mediante cromatografía de líquidos (HPLC) acoplado a un detector de espectrometría de masas de alta resolución y un método usando cromatografía de gases (GC) acoplado a un detector de espectrometría de masas para la determinación de catabolitos derivados de compuestos fenólicos presentes en orina.

- Objetivo específico 5.2: Comparar los dos métodos cromatográficos teniendo en cuenta los procesos de preparación y extracción de muestra.

Esta Tesis Doctoral está estructurada como una recopilación de artículos científicos que comprende 1 revisión bibliográfica y 7 publicaciones de carácter experimental en revistas indexadas. El trabajo experimental se ha realizado en el Área de Nutrición y Bromatología de la Facultad de Farmacia (Universidad de Sevilla) y en el Área de Alimentación y Salud del centro IFAPA-Alameda del Obispo (Córdoba).

De esta forma, los estudios llevados a cabo en la Universidad de Sevilla se han desarrollado gracias a la financiación del proyecto "Determinación de Melatonina en Uvas, Vinos y Otros Alimentos de Andalucía" (P07-AGR-02480) otorgado por la Consejería de Economía, Innovación y Ciencia de la Junta de Andalucía y del proyecto "Evaluación de la Calidad y Seguridad de una Nueva Bebida Obtenida a partir de Fresa no apta para Comercialización" (AGL2010-22152-C03-01) otorgado por el Ministerio de Ciencia e Innovación. Del mismo modo, los estudios realizados en el IFAPA-Alameda del Obispo se han llevado a cabo gracias a la financiación del proyecto "Caracterización de alimentos y nuevos productos elaborados: potencial saludable, organoléptico y trazabilidad alimentaria. Estrategias de diversificación y reclamo competitivo" (PP.AVA.AVA201601.20) otorgado por el Instituto de Investigación y Formación Agraria y Pesquera (Consejería de Agricultura, Pesca y Desarrollo Rural, Junta de Andalucía).

CAPÍTULO I

Resumen

Este primer capítulo está dedicado a una revisión bibliográfica sobre las tendencias más recientes en la determinación de aminas biógenas en bebidas fermentadas, centrándose fundamentalmente en la novedad, mejora y optimización de los métodos analíticos desde 2010 hasta 2016.

Las aminas biógenas son una familia de moléculas presente en los alimentos con un riesgo potencial sobre la salud, especialmente en los consumidores sensibles. Actualmente, no existen unos límites máximos permitidos en alimentos para estas biomoléculas en la legislación europea, a excepción de la histamina en los productos pesqueros. Principalmente, estos compuestos son sintetizados por la actividad microbiana durante el almacenamiento y procesamiento de los alimentos. Por todo ello, junto a la preocupación económica asociada, hace que sea necesario controlar la concentración de aminas biógenas en los alimentos. De esta forma, en la bibliografía se puede encontrar una gran cantidad de artículos centrados en la determinación de aminas biógenas en diferentes productos alimenticios, especialmente en bebidas fermentadas.

A lo largo del capítulo se describen y discuten los diferentes métodos para la preparación tratamiento de muestras (incluida la derivatización), las técnicas analíticas más importantes y las aplicaciones más frecuentes referenciados. Aunque las aminas biógenas se han determinado en el vino y otras bebidas fermentadas durante décadas, las nuevas técnicas y avances han permitido mejorar la precisión y la sensibilidad de los métodos analíticos, superando los desafíos planteados por las matrices complejas, como la dificultad para ser detectados por la presencia de otros compuestos similares o la alta variabilidad intrínseca de las muestras. De este modo, se han revisado los diferentes propósitos de la determinación de aminas biógenas, tales como seguridad alimentaria, proceso de producción o investigación de microbiología alimentaria, además de las técnicas analíticas más empleadas.

Artículo 1

Recent trends in the determination of biogenic amines in fermented beverages – A review.

***José Luis Ordóñez**, Ana Maria Troncoso, Maria Del Carmen García-Parrilla, Raquel Maria Callejón.*

Analytica Chimica Acta
939 (2016) 10-25

Q1; 7/76 (JCR-ISI, Analytical Chemistry, 2016)
Impact Factor: 4.950

Analytica Chimica Acta 939 (2016) 10–25

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Review

Recent trends in the determination of biogenic amines in fermented beverages – A review

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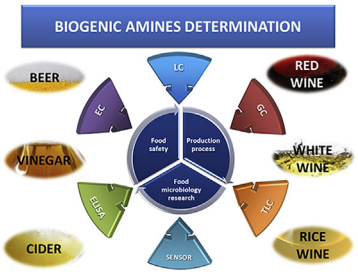
HIGHLIGHTS

- A critical review on analytical methods for BA in fermented beverages is presented.
- Recent sample treatments and analytical techniques are described and discussed.
- A previous derivatization is needed in most liquid chromatographic methods.
- BA determination is related to food safety, production process or microbiology research.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 7 April 2016
Received in revised form
26 July 2016
Accepted 28 July 2016
Available online 3 August 2016

Keywords:
Biogenic amines
Histamine
Tyramine
Derivatization
Sample preparation
Fermented product

ABSTRACT

Biogenic amines (BA) are generally considered as a food hazard, even though there is not a threshold for these biomolecules in the European legislation, except for histamine in fishery products. These compounds are formed during the storage and processing of certain foods through microbiological activity, and when present in high concentrations, could have toxicological effects, causing health problems in consumers, especially to sensitive persons. This fact, in addition to the economical concern involved, makes it necessary to control the amounts of biogenic amines in foods. For all these reasons, literature on biogenic amines in different food products, especially in fermented beverages, is extensive. This review provides an overview of the most recent trends in the determination of biogenic amines in fermented beverages focusing on novelty, improvement and optimization of analytical methods. Hence, the different sample treatment procedures (including derivatization), the most important analytical techniques and the most frequent applications are described and discussed. Although biogenic amines have been determined in wine and other fermented beverages for decades, new advancements and technical possibilities have allowed to increase the accuracy and sensitivity of analytical methods, in order to overcome the challenges posed by the complex matrices and their high intrinsic variability. Thus, the different purposes of BA determination (food safety, production process or food microbiology research) and the most widely employed analytical techniques have been reviewed.

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http://dx.doi.org/10.1016/j.aca.2016.07.045
0003-2670/

Contents

1. Introduction

The control of biogenic amines in foods is gaining importance in order to monitor production processes or to know their quality and freshness, but mainly to monitor food safety [1,2]. Thus, the number of analyses of these compounds has increased during the last years. They are mainly synthetized in foods and beverages by decarboxylation of amino acids, although some amines can be formed by amination and transamination of aldehydes and ketones [3].

The concentrations (mg kg^{-1} or L^{-1}) of the main BA in a wide range of products are summarized in Table 1. In general, the highest concentrations of these compounds have been found in fermented products, which show a significantly higher amount than non-fermented foods. Thus, histamine reached high levels in sufu, fish sauce, sausages and ripened cheeses (Table 1). Specially, a concentration of histamine of 10000 mg kg^{-1} was detected in spoiled fish [1]. Tyramine has been detected in high amounts in cheese samples, mainly in ripened cheese (Table 1). This could have an effect on health, due to its capacity to potentiate sympathetic cardiovascular activity by releasing noradrenaline, which is named 'cheese reaction' [53]. Regarding putrescine and cadaverine, they have been detected in significant concentrations in fermented meat and fish (Table 1). BA presence in foods is closely related to microbial activity. Thus, lactic acid bacteria are the main producers, although some species of yeast or bacteria of the family *Enterobacteriaceae* could synthetize them [54].

BA can produce a wide range of toxicological effects [1] being histamine and tyramine the main BA, regarding their toxic effect. Histamine is the most widely studied amine due to its ability to produce headaches, hypotension and digestive problems, while tyramine is often associated with migraine and hypertension [54]. Recently, Linares et al. [55] proved that tyramine was more cytotoxic than histamine on an *in vitro* model of the human intestinal epithelium. Thus, they observed that tyramine caused a cell necrosis, whilst histamine induced apoptosis. On the other hand, other polyamines such as putrescine and cadaverine have a lower pharmacological activity; however, they could interact with the amine oxidases and potentiate the effects of histamine and tyramine. Besides, these polyamines can react with nitrite to form carcinogenic nitrosamines [56].

Although BA have been described as having a certain potential toxicity, the maximum histamine level is only regulated in fishery products, at 50 mg kg^{-1} by the US Food and Drug Administration (FDA), and at 100 mg kg^{-1} by the European Community [57].

However, the European Food Safety Authority (EFSA) has released a scientific opinion where it remarks the risks associated with the increase of BA in fermented products [1]. This document concluded by stating the importance of controlling these compounds in food, as well as validating analytical methods for different types of matrices. On the other hand, some countries in Europe recommended establishing limits for histamine in wine [Germany (2 mg L^{-1}), Belgium (5–6 mg L^{-1}), and France (8 mg L^{-1})] [58,59]. Switzerland also established a legal threshold of 10 mg L^{-1} for histamine, which was rejected afterwards [41]. In this context, the International Organization of Vine and Wine (OIV) published, in the Compendium of International Methods of Analysis of Wines and Musts, two chromatographic methods to determine BA in order to standardize methods of analysis, thus helping to facilitate international trade [60]. Regarding beer, the *Nutritional codex* of the *Slovak Republic* recommended a maximum tolerable limit for histamine of 20 mg kg^{-1} [61].

According to Scopus database, a significant number of papers dealing with BA determination in food have been published since 2010. Thus, there are 1037 articles related to agricultural and microbiology areas. As shown in Fig. 1, the main food items analyzed were meat, fish, cheese and wine. Taking the fermented beverages and condiments as a group (wine, beer and vinegar), and together with fruit juices, they accounted for a 22% of the total research articles published in the aforementioned period.

Specifically, fermented beverages are constituted by a complex matrix that increases the difficulty of analyses and interferes with the results. The complexity of the varied food matrices is a critical aspect to be taken into account when obtaining adequate recoveries for all BA [1]. Regarding analytical determination, an array of methods such as high performance liquid chromatography (LC), ultra performance liquid chromatography (UPLC), gas chromatography (GC), thin-layer chromatography (TLC), ion-pair liquid chromatography (IPLC), capillary electrophoresis (CE), sensors and ELISA, among others, have been used for these compounds.

The literature includes some review articles focused on biogenic amines in foods, but none of them considers thoroughly the sample preparation, as well as all the analytical techniques employed for the determination of these compounds in food products – most of them only consider chromatographic methods. On the other hand, despite the number of works regarding biogenic amines in wine, beer and other beverages, there is currently no review article which compares and discusses the analytical methods for determining biogenic amines in these matrices.

For all the above, this review provides an overview of the most recent trends in the determination of BA in wine, beer, cider, fermented beverages and condiments, focusing on novelty, improvement and optimization of analytical methods from 2010 to present. Hence, the different sample treatment procedures (including derivatization), the most important innovations and improvements in the analytical techniques and the most frequent applications are

Table 1
Content of main biogenic amines in foods and beverages (mg kg^{-1} or L^{-1}).

	His	Tyr	Put	Cad	Ref.
Fermented Food					
Vegetables					
Cabbage	1.0	18.6	11.6	7.6	[4]
Soybeans/tofu	n.d.-5.8	n.d.	0.7–5.0	n.d.-3.4	[5,6]
Fruit and cereal					
Grapes	n.d.-5.8	n.d.-2.4	n.d.-8.0	n.d.-3.4	[7]
Apples	n.d.	n.d.	5.1	–	[8]
Barley	n.d.	8.0–18.9	7.6–21.0	0.5–1.1	[9]
Meat					
Pork meat	0.9–2.3	n.d.-29.0	n.d.-66.5	1.0–145.4	[10]
Chicken meat	0.2	n.d.-221.6	n.d.-45.9	n.d.-33.5	[11,12]
Beef meat	0.4–7.4	n.d.-17.4	n.d.-202.5	n.d.-221.4	[10,11]
Fish and mollusc					
Salted anchovies	n.d.-2.0	n.d.-22.3	0.1–8.0	0.1–12.0	[13,14]
Fresh mackerel	9.3–12.4	n.d.-0.4	0.2–2.0	1.2–6.6	[15]
Canned tuna	n.d.-110.3	n.d.-48.6	n.d.-116.5	n.d.-103.3	[16]
Octopus	1.3–9.1	n.d.-14.5	2.8–94.1	0.1–164.0	[17]
Beverages					
Milk	n.d.-0.7	n.d.	n.d.	n.d.-0.1	[18,19]
Brewed coffee	n.d.-1.6	n.d.-19.7	0.4–2.3	0.2–9.1	[20,21]
Orange juice	n.d.-0.04	n.d.-0.06	0.1–2.2	–	[22,23]
Others					
Honey	n.d.-3.8	n.d.	n.d.	n.d.	[24]
Chocolate	0.3–2.0	3.1–8.1	0.8	0.8	[25,26]
Fermented Food					
Vegetables					
Sauerkraut	2.1–37.0	26.5–94.4	32.1–122	17.1–41.1	[4,25]
Sufu	n.d.-730.0	n.d.-1730	0.5–316.9	0.6–85.8	[5,27,28]
Soy sauce	n.d.-85.0	0.9–80.5	7.0–108.0	0.2–85.0	[5,29]
Meat					
Chorizo	n.d.-4.5	3.1–186.1	n.d.-178.3	n.d.-52.0	[30,31]
Sausages	n.d.-514.5	n.d.-509.9	n.d.-505.3	n.d.-689.8	[31–33]
Fish					
Fish sauce	n.d.-729	n.d.-1178	n.d.-1257	n.d.-1429	[29,34]
Dairy products					
Yogurt	n.d.-13	n.d.-6.3	n.d.-26.1	n.d.-4.3	[18,25,35]
Butter	–	4.6–5.0	n.d.	n.d.	[35]
Unripened cheeses	n.d.-6.7	n.d.-2.7	n.d.-0.9	n.d.-8.9	[35–37]
Ripened cheeses	n.d.-337.9	n.d.-2520	n.d.-105.8	n.d.-774.5	[35–37]
Kefir milk	n.d.-4.0	n.d.-9.8	0.4–14.3	n.d.-2.2	[35,38]
Fermented Beverages					
Red wine	0.5–27.0	0.1–37.3	2.9–122	n.d.-3.3	[39–41]
White wine	n.d.-3.4	n.d.-6.8	0.8–12.8	n.d.-2.5	[42–44]
Rice wine	n.d.-72.1	n.d.-41.4	n.d.-32.3	n.d.-63.5	[45,46]
Beer	n.d.-0.3	0.4–5.9	2.1–12.8	0.2–1.4	[47,48]
Vinegar	n.d.-0.3	n.d.-0.2	n.d.-3.2	n.d.-0.1	[49,50]
Cider	n.d.-6.9	n.d.-5.0	n.d.-12.3	–	[51]
Gluconic fermented	n.d.	n.d.	n.d.	n.d.	[52]

Ref.: References; n.d.: non detected; -: non determined
Analytes: His: Histamine; Tyr: Tyramine; Put: Putrescine; Cad: Cadaverine.

described and discussed. The review focuses on BA which have a greater toxicological potential and health concern; therefore, histamine, tyramine, putrescine and cadaverine have been selected for discussion. On the other hand, the different purposes of BA determination (food safety, production process monitoring or food microbiology research) and the most widely employed analytical techniques have been evaluated. Although BA have been determined in wine and other fermented beverages for decades, new advancements and technical possibilities have allowed to increase the accuracy and sensitivity of analytical methods, in order to overcome the challenges posed by the complex matrices and their high intrinsic variability.

2. Analytical methods

2.1. Liquid chromatography

The determination of BA in fermented products is not simple, due to the variety of their chemical structures, as well as their presence at low concentrations in complex matrices (Table 1) [49]. The most commonly employed technique is LC using C18 reverse-phase columns, due to its high resolution and sensitivity [62]. A previous preparation is usually required in order to remove other compounds that may interfere in the chromatography analysis, or in order to concentrate the analytes of interest.

Moreover, the determination of BA by LC usually needs a chemical derivatization, because these compounds do not have adequate absorption properties in the visible, ultraviolet or fluorescence wavelength ranges [63]. Derivatization reduces the polarity of BA, thus improving the separation by reverse phase mode and C18 columns, and making them more sensitive towards MS detector than their correspondent underivatized amines [25,57,64].

2.1.1. Sample preparation and extraction

In general, different types of sample preparation, such as filtration, degasification, the addition of compounds to remove

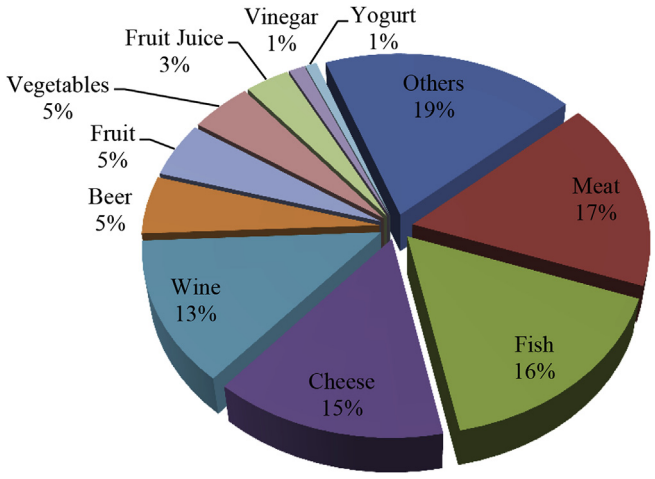

Fig. 1. Percentage breakdown of biogenic amine researches related to food products published since 2010.

substances and extraction have been employed (Table 2). Regarding the addition of compounds, polyvinylpyrrolidone (PVP) has been extensively used, as it could remove the substances that interfere in the derivatization reaction (polyphenols and related substances present in fermented beverages). Therefore, it was proved to be useful in methods that employed reagents with low specificity, such as dansyl chloride and benzoyl chloride [117]. Besides, the addition of acid or organic solvents, such as 5-sulfosalicylic acid [110] or trichloroacetic acid (TCA) [87], allowed to precipitate the proteins present in the samples.

On the other hand, several extraction methods have been widely used to concentrate the analytes and clean up samples, thus improving limits of detection and avoiding interferences. Therefore, some traditional extraction techniques, such as liquid-liquid extraction (LLE), are still applied in food samples treatment using a wide range of organic solvents such as toluene, chloroform, dichloromethane, ethyl acetate, ethanol or isohexane (Table 2). These techniques have several disadvantages, as they need a large volume of hazardous organic solvents as well as being time-consuming procedures, and a considerable volume of sample is often required for trace analysis. For these reasons, new LLE-based techniques have evolved by using liquid-phase micro-extractions (LPME). Table 2 shows other BA extraction techniques based on the LLE, such as salting-out assisted liquid-liquid extraction (SALLE), dispersive liquid-liquid microextraction based on solidification of floating organic droplets (DLLME-SFO), and vortex assisted surfactant-enhanced emulsification liquid-liquid microextraction (VSLLME). These techniques show several advantages, as they employ less toxic organic solvents, reduce time extraction, prevent sample loss and provide a more precise and accurate extraction [46,91,99]. Regarding VSLLME, a significant improvement in LODs (from 294–871 μg L^{-1} to 1–2.6 μg L^{-1}) was observed as compared with direct samples injection [99].

Another alternative to avoid problems associated to the organic solvents used in LLE was the extraction using ionic liquids (IL). This technique uses relatively large organic cation and inorganic anion solutions and provides a medium that improves the derivatization of BA [95,118]. Thus, room-temperature ionic liquids (RTILs), which integrated derivatization, extraction and preconcentration of BA, was applied in wine samples, obtaining very low LODs (1–2 μg L^{-1}) and a high extraction efficiency (85–96%) (Table 2). Besides, Huang et al. [83] used the ionic liquid-based ultrasonic-assisted liquid-liquid microextraction method (IL-UALLME), which was a new technique based on IL. Its main advantage was that it had a very short extraction time (1 min).

However, the most common extraction method for BA determination in beverages is solid phase extraction (SPE) [119]. However, it has also some disadvantages, such as being time-consuming or relatively expensive. An alternative is solid phase microextraction (SPME), which reduces the solvent volume. Basheer et al. [23] elaborated a new SPME with hydrazone-based ligands with good LODs between 3.82 and 31.30 ng L^{-1} (Table 2). In addition, an innovative molecularly imprinted solid phase extraction (MISPE) with specific affinity towards histamine was elaborated and applied in wine samples with successful recoveries (93–99%) [66]. The extraction method was very reproducible and accurate, but the chromatography method was too long (40 min) for just one BA, and it had a high LOD (0.09 mg L^{-1}).

Alternatively, Gas-diffusion microextraction (GDME) was another extraction technique employed to determine volatile aliphatic BA (methylamine, dimethylamine and ethylamine) in fermented beverages [108].

2.1.2. Derivatization reaction

Derivatization reaction can be performed before (pre-column methods) or after (post-column methods) the chromatographic analysis. Although pre-column methods have been commonly used, these are usually more susceptible to matrix effects than post-column methods [120]. Most derivatization reagents have an optimum pH of reaction higher than 7.5 [3]. This fact may affect wine matrices, since they show a high concentration of organic acids, such as tartaric and malic acids, their pH being sensibly lower [120]. Solutions with buffer capacity are usually used to avoid this drawback; however, slight pH variations, or samples with very low pH, like some wines or vinegars, may affect the derivatization reaction [120]. As far as we know, only a few studies have been developed to assess the quantitative formation of derivatives with regard to different buffer solutions [89].

Most works related to BA in fermented beverages and condiments use a derivatization reagent prior to the analysis. The most used reagents during the last years are dansyl chloride (DNS-Cl), *o*-Phthalaldehyde (OPA), benzoyl chloride, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC), diethyl ethoxymethylenemalonate (DEEMM) or 4-chloro-3,5-dinitrobenzotrifluoride (CNBF) as reported by Peña-Gallego et al. [3], Önal et al. [121], and Callejón et al., [122]. They react with amine groups and have been indistinctively employed for the determination of amino acids and BA [122]. Thus, most of them could react with primary and secondary amines, except for OPA, which it is not able to react with secondary amines [3]. However, amino acids are usually several orders of magnitude higher than BA in fermented products, thus hindering their detection. In order to solve this inconvenience, more concentrated reagent solutions have been commonly used; however, new problems may arise due to interferences or degradation of reagent excess [120].

Among these reagents, the most commonly employed is DNS-Cl. This compound produces stable derivatives which are detected by UV–visible (UV–vis) or fluorescent spectroscopy [3]. However, DNS-Cl is a non-specific reagent, since it can react with other substances such as phenols or aliphatic alcohols [117]. Hence, sample treatments are usually employed to remove interferences (Table 2). This reagent has been employed in different matrices such as beer, orange juices or wine (Table 2). On the other hand, the derivatization time may vary, and it depends on temperature, as shown in Table 2. In general, those methods that used a fluorescent detector were more sensitive than those with a UV–vis detector [88,89,96]. However, the best LODs were obtained by Tameem et al.

Table 2
Biogenic amine determination by chromatographic methods.

Separation techniques/ Der. reagent	Matrix	BA and other compounds	Sample prep.	Extraction method	Der. conditions	Column	Mobile phase	Run time	Detection	LOD (μg L^{-1})	Rec. (%)	Ref.
LC												
–	Wine	His	–	–	–	Cogent Diamond Hydride (DH) (75 × 4.6 mm, 4 μm)	A: H_2O/0.1% FA, B: ACN/0.1% FA	10 min	UV–vis λ: 220 nm	LOQ: 2	–	[65]
	Wine	His	–	–	–	Cogent Diamond Hydride (DH) (150 × 2.1 mm, 4 μm)	A: H_2O:2-propanol (50:50)/0.1% FA, B: ACN/0.1% FA	10 min	MS (ESI +)	LOQ: 0.2	–	[65]
	Wine	His	MISPE	–	–	Agilent Eclipse XDB-C18 (150 × 4.6 mm, 5 μm)	A: 5 mM AF buffer/5 mM PFHA in H_2O, B: 5 mM AF buffer/5 mM PFHA in MeOH	40 min/ RT	UV–vis λ: 212 nm	90	93 –99	[66]
	Beer	His	Degassed	–	–	Silica capillary nanoemitter (5±1 μm)	MeOH:H_2O (50:50)/0.1% FA	several s/180 °C	MS-MS (nanoEESI)	20	–	[67]
AQC	Vinegar and Wine	His, Tyr, Put, Cad, Met, Agm, Phe, Spm, Spmd	–	SPE	10 min/55 °C	Luna C18 (250 × 4.6 mm, 5 μm)	A: 140 mM NaAc/17 mM Tea, (pH 5.05), B: MeOH	48 min/ 65 °C	Fl λex/em: 250/395 nm	7–26	65 –106	[49,68]
	Wine	His, Tyr, Put, Cad, Spm, Spmd, Phe, Dimet, Hex, Diety, Ety, Pyr, Isob, Isop, Amy	–	–	10 min/55 °C	RP column (300 × 3.9 mm, 4 μm)	A: 140 mM NaAc/17 mM Tea, (pH 5.05), B: MeOH	32 min/ 65 °C	Fl λex/em: 250/395 nm	–	73 –105	[69]
	Wine	His, tyr, Put, Cad	–	–	10 min/55 °C	Phenomenex Kinetex (PFP) (100 × 4.6 mm, 2.6 μm)	A: 140 mM NaAc/17 mM Tea, (pH 5.05), B: MeOH	25 min/ 65 °C	Fl λex/em: 250/395 nm	120 –340	–	[70]
	Wine	His, Tyr, Put, Cad, Phe, Spm, Spmd	–	–	10 min/55 °C	RP column (300 × 3.9 mm, 4 μm)	A: 140 mM NaAc/17 mM Tea, (pH 5.05), B: MeOH	32 min/ 65 °C	Fl λex/em: 250/395 nm	–	–	[71]
Benzoyl chloride	Non-alcoholic beers	His, Tyr, Put, Cad, Try, Agm, Phe, Spm, Spmd	Degassed and PVP	LPME (Derivatized BA extraction)	20 min/RT	Waters symmetry C18 (150 × 3.9 mm, 5 μm)	A: MeOH, B: H_2O	30 min	UV–vis λ: 254 nm	20–90	87 –98	[72,73]
	Rice wine	His, Tyr, Put, Cad, Try, Ser, Phe, Spm, Spmd	0.1 M HCl	–	60 min/30 °C	Inertsil ODS-3 (250 × 4.6 mm, 5 μm)	A:10 mm AmAc, B: ACN	40 min/ 30 °C	UV–vis λ: 254 nm	250 –2000	89 –114	[74]
	Şalgam	His, Tyr, Put, Cad, Met, Agm, Try, Phe, Spm, Spmd	–	–	15 min/25 °C	Hichrom C18 (300 × 3.9 mm, 10 μm)	A: 0.05 M Ac buffer:MeOH (60:40), B: MeOH	31 min/ 20 °C	UV–vis λ: 254 nm	200 –2500	80 –100	[75]
	Sorghum brew and Sour maize beverage	His, Tyr, Put, Cad	TCA (6%)	Diethyl ether (Derivatized BA extraction)	40 min/25 °C	C18 Waters Spherisorb ODS-2 (150 × 4.60 mm, 3 μm)	A: ACN, B: H_2O	6.5 min	UV–vis λ: 254 nm	–	–	[76]
	Wine	His, Tyr, Put, Cad, Phe	–	Chloroform (Derivatized BA extraction)	20 min/RT	Luna C18 (250 × 4.6 mm, 5 μm)	A: H_2O, B: ACN	15 min/ 22 °C	UV–vis λ: 254 nm	45–57	90 –99	[77]
	Wine	His, Tyr, Put, Cad, Try, Spm, Spmd, Phe, Eth, Mpa, 3-Mba, 2-Mba, 3-Mtpa; Other: β-ala	N_2 liquid and TCA (10%)	DCM (Derivatized BA extraction)	120 min/ RT	Phenomenex Aqua C18 125 Å (1.5 cm × 0.2 cm, 5 μm)	A: H_2O, B: ACN/0.1% FA	48 min/ 30 °C	MS/MS (ESI +)	LOQ: 0.05 μg/kg	98 –104	[25]
	Wine, Rice wine and beer	His, Tyr, Put, Cad, Try, Phe, Dap, Spm, Spmd	–	DLLME-SFO (Derivatized BA extraction)	30 min/30 °C	Gemini C18 (150 × 4.6 mm, 5 μm)	A: H_2O, B: MeOH	32 min/ 30 °C	UV–vis λ: 254 nm	5–10	61 –147	[46]
CNBF	Rice wine	His, Tyr, Put, Cad, Try, Phe, Spm, Spmd	–	–	30 min/60 °C	Zorbax Eclipse XDB-C18 (250 × 4.5 mm, 5 μm)	A: ACN, B: 0.1 M Ac buffer (pH 6.2)	22 min/ 35 °C	UV–vis λ: 254 nm	10 –400	–	[78]
	Wine	His, Tyr, Phe, Try	Degassed	Ethyl acetate	120 min/ RT	Gemini-NX C18 (250 × 4.6 mm, 5 μm)	A: MeOH:H_2O (80:20), B: MeOH	23 min/ 25 °C	UV–vis λ: 254 nm	20–30	97 –102	[79]
d_0-/d_3-MASC	Rice wine	His, Tyr, Put, Cad, Try, Phe, Hex	TCA (5%)	–	15 min/55 °C	Eclipce Plus C18 (50 × 2.1 mm, 1.8 μm)	A: H_2O:ACN (95:5)/0.1% FA, B: ACN/0.1% FA	5 min/ 30 °C	MS/MS (ESI +)	8.65 –12.7	95 –105	[80]
DEEMM	Wine	His, Tyr, Put, Cad, Met, Phe, Eth, Isoa	–	–	30 min/RT	ACE HPLC (5C18-HL) (250 × 4.6 mm, 5 μm)	A: 25 mM Ac buffer (pH 5.8)/0.02% Na azide, B: ACN:MeOH (80:20)	57 min/ 16 °C	UV–vis λ: 280 nm	100 –500	95 –101	[39]
	Wine	His, Tyr, Put, Cad, Agm, Try, Phe, Spmd, Isoa; Others: Aa	–	–	30 min/RT	–	A: 25 mM Ac buffer (pH 5.8)/0.02% Na azide, B: ACN:MeOH (80:20)	85 min/ 16 °C	UV–vis λ: 280 nm	–	–	[81]

	Wine	His, Tyr, Put, Cad, Phe, Spm, Spmd, Agm, Eth, Ser; Others: Aa	–	–	30 min/RT	ZORBAX SB-C18 Rapid Resolution HT (50 × 3.0 mm,1.8 μm)	A: 25 mM Ac buffer (pH 5.8)/0.02% Na azide, B: ACN	30 min/ 16 °C	UV–vis λ: 280 nm	30 –100	80 –118	[82]
	Gluconic acid	His, Tyr, Put, Cad, Agm, Try, Phe, Spmd; Others: Aa	–	–	30 min/RT	LiChroCART® 250-4 LiChrospher® 100 RP-18 (250 × 4.6 mm, 5 μm)	A: 25 mM Ac buffer (pH 5.8)/0.02% Na azide, B: ACN:MeOH (80:20)	85 min/ 45 °C	UV–vis λ: 280 nm	50 –100	–	[52]
DMQC-Osu	Beer	Tyr, Phe, Oct	–	IL-UALLME (Derivatized BA extraction)	40 min/20 °C	Eclipse XDB-C18 (150 × 4.6 mm, 5 μm)	MeOH:H_2O (60:40)	16 min	Fl λex/em: 326/412 nm	0.25 –50	90 –116	[83]
DNS-Cl	Beer	His, Tyr, Put, Cad, Try, Phe, Spm, Spmd	Degassed and 0.6 M PCA	–	20 h/RT	Zorbax Eclipse XDB-C18 (150 × 4.6 mm, 3.5 μm)	A: H_2O, B: ACN	23 min/ RT	UV–vis λ: 254 nm	–	–	[84]
	Beer	His, Tyr, Cad, Try; Others: precursor Aa	Degassed	SPE	40 min/65 °C	LiChrospher C18e (250 × 4 mm, 5 μm)	A: ACN, B: 9 mM AF buffer (pH 3.40)	50 min 40 min 40 min	UV–vis λ: 254 nm MS(APCI +) MS (ESI +)	14–67 7–58 10–54	>92	[47]
	Beer	His, Tyr, Put, Cad, Try, Phe, Spm, Spmd, Agm	2 M NaOH and $NaHCO_3$ solution	–	45 min/40 °C	Waters dc18 (250 × 4.6 mm, 5 μm)	A: ACN, B: H_2O	26 min/ 40 °C	UV–vis λ: 254 nm	–	–	[85]
	Beer	His, Tyr, Put, Cad, Phe, Try, Spm, Spmd	Desgassed	n-Butanol: Chloroform (1:1)	60 min/40 °C	Restek Ultra Aqueous C18 (150 × 4.6 mm, 3 μm)	A: ACN, B:P buffer (pH 7), C:H_2O	25 min	UV–vis λ: 254 nm	7–23	20 –70	[86]
	Orange juices	His, Tyr, Put, Cad, Try, Phe, Spmd	5 mL 0.1 M HCl	SPE, sorbent material based on hydrazone ligand	10 min/70 °C	C18 ODS Hypersil (250 × 4.5 mm, 5 μm)	ACN:H_2O:MeOH (60:25:15)	18 min/ RT	UV–vis λ: 254 nm	0.02 –0.06	67 –98	[29]
	Orange juices	His, Tyr, Put, Cad, Try, Spmd	–	SPME (Derivatized BA extraction)	10 min/70 °C	COSMOSIL 5C18-MS-I (250 mm × 4.6 mm)	ACN:H_2O (70:30)	20 min	UV–vis λ: 254 nm	0.004 –0.031	80 –95	[23]
	Rice wine	His, Tyr, Put, Cad, Ser; Others: Aa	–	–	10 min/70 °C	Waters-Atlantisd C18 (250 × 4.6 mm, 5 μm)	MeOH:H_2O (55:45)	16 min/ 30 °C	UV–vis λ: 254 nm	–	–	[45]
	Rice wine	His, Tyr, Put, Cad, Try, Phe, Spm, Spmd	TCA (5%)	–	–	Agilent C18 (150 × 4.6 mm, 5 μm)	A: MeOH, B: H_2O	45 min/ 30 °C	UV–vis λ: 254 nm	100 –190	92 –106	[87]
	Wine	His, Tyr, Put, Cad, Phe, Spm, Spmd	PVP	–	60 min/47 °C	Waters C18 YMC-Pack ODS-A (150 × 4.6 mm, 5 μm)	A: MeOH, B: H_2O	45 min/ 45 °C	Fl λex/em: 330/520 nm	1–90	>76	[88]
	Wine	His, Tyr, Put, Cad, Phe, Spm, Spmd	PVP	–	60 min/60 °C	Waters C18 YMC-Pack ODS-A (150 × 4.6 mm, 5 μm)	A: H_2O, B: ACN	40 min/ 40 °C	UV–vis λ: 254 nm	90 –300	>78	[89]
	Wine	His, Tyr, Put, Cad, Phe, Spm, Spmd, Ety, Isoa	–	–	60 min/40 °C	Waters Spherisorb C18 S3ODS-2 (150 × 4.6 mm, 3 μm)	A: ACN, B: P buffer (pH 7), C: H_2O	15 min	UV–vis λ: 254 nm	100 –500	>70	[44]
	Wine	His, Tyr, Put, Cad, Met, Phe, Spm, Spmd, Eth, Isoa, Ety	–	–	45 min/40 °C	Supelcosil LC-18 (250 × 4.6 mm, 5 μm)	A: ACN, B: H_2O	35 min/ 25 °C	UV–vis λ: 254 nm	20 –100	71 –99	[90]
	Wine	His, Tyr, Put	–	–	60 min/40 °C	PerfectSil Target ODS-3 (250 × 3.0 mm, 5 μm)	A: 0.1 M Tris buffer (pH 8.5): H_2O (1:3), B: ACN	43 min/ 35 °C	Fl λex/em: 254/510 nm	–	–	[40]
	Wine	His, Put, Cad, Phe, Met, Isop, Spmd, Eth, Dimet	–	SALLE (Derivatized BA extraction)	25 min/20 °C	Phenomenex Gemini C18 (250 × 4.60 mm, 5 μm)	A: ACN, B: 0.01 M Ac buffer (pH 4.0)	45 min	Fl λex/em: 320/523 nm	3–220	–	[91]
	Wine	His, Tyr, Put, Cad, Met, Agm, Phe, Spm, Spmd, Isope, Dop, Ety, Try; Others: Aa	–	–	30 min/40 °C	Phenomenex Gemini C18 (150 × 4.60 mm, 3 μm)	A: Ac buffer (pH 4.1)/ACN, B: ACN	64 min/ 25 °C	Fl λex/em: 293/492 nm	4–60	95 –103	[43]
	Wine	His, Tyr, Put, Cad, Phe, Spm, Spmd, Isoa, try	–	–	60 min/55 °C	Phenomenex Synergi polar RP 80A (150 × 4.60 mm, 4 μm)	A: Ethanol:ACN:H_2O: Tris buffer (pH 8) (20:30:47:3), B: Ethanol:ACN:H_2O: Tris buffer (pH 8) (45:45:9.8: 0.2)	43 min/ RT	UV–vis λ: 220 nm	70	68 –111	[41]

(*continued on next page*)

Table 2 (*continued*)

Separation techniques/ Der. reagent	Matrix	BA and other compounds	Sample prep.	Extraction method	Der. conditions	Column	Mobile phase	Run time	Detection	LOD (μg L^{-1})	Rec. (%)	Ref.
	Wine	His, Tyr, Put, Cad, Spm, Spmd, Try, Dap	–	Toluene (Derivatized BA extraction)	60 min/60 °C	Phenomenex Gemini C18 (150 × 4.60 mm, 5 μm)	A: ACN, B: H_2O	21 min	Fl λex/em: 360/510 nm	–	–	[92,93]
	Wine	His, Tyr, Put, Cad, Agm, Met, Spm, Spmd, Try, Phe, Eth	–	Diethyl ether (Derivatized BA extraction)	5 min/60 °C	Waters C18 Symmetry Shield RP18 (150 × 4.6 mm, 3.5 μm)	A: ACN, B: H_2O	–	UV–vis λ: 254 nm	–	–	[94]
	Wine and China yellow wine	Tyr, Put, Cad, Try, Phe, Spm	PVP	RTIL (Derivatized BA extraction)	20 min/RT	Agilent Zorbax SB-C18 (250 × 4.6 mm, 5 μm)	A: ACN, B: H_2O	25 min/ 30 °C	UV–vis λ: 254 nm	1–2	82 –118	[95]
	Wine and Fruit nectar	His, Tyr, Put, Cad, Agm, Phe, Met, Spm, Spmd, Ser, Ety	–	0.4 M PCA (nectar)	60 min/45 °C	Kinetex C18 core-shell particle (100 × 4.6 mm, 2.6 μm)	A: ACN, B: H_2O	13 min/ 50 °C	UV–vis λ: 254 nm	13 –112	96 –104	[96]
									Fl λex/em: 320/523 nm	2–23		
	Fermented soybean paste (Doenjang)	His, Tyr, Put, Cad, Agm, Phe, Try, Spm, Spmd	–	0.4 M PCA	45 min/40 °C	C18 supelco (250 × 4.6 mm, 5 μm)	A: 0.1 M AmAc, B: ACN	35 min	UV–vis λ: 254 nm	–	–	[97]
EAC	Beer	His, Tyr, Put, Cad, Hex, Phe, Spd, Spmd; Others: Estrogens	0.1 M HCl	–	6 min/45 °C	Hypersil BDS C18 (200 × 4.6 mm, 5 μm)	A: H_2O:ACN (75:25)/0.1% AF buffer, B: ACN	50 min/ 36 °C	Fl λex/em: 270/430 nm	0.27 –0.67	–	[98]
FMOC	Wine and Beer	His, Tyr, Cad, Try, Spmd	Degassed	VSLLME (Derivatized BA extraction)	30 min/RT	Waters symmetry C18 (150 × 3.9 mm, 5 μm)	A: ACN, B: H_2O/1% AcAc	30 min	UV–vis λ: 265 nm	1–2.6	83 –113	[99]
OPA	Must	His, Tyr, Put, Cad, Agm, Eth, Ety	–	–	–	Alltima C18 (200 × 4.6 mm, 5 μm)	A: 1.2 g NaAc/500 mL H_2O/0.09 mL TEA/1.5 mL THF, B: 1.1 g NaAc/100 mL H_2O/200 mL ACN/200 mL MeOH	60 min	Fl λex/em: 340/450 nm	10–20	–	[7]
	Wine	His, Tyr, Put Cad, Phe, Isope	–	Ethanol	–	Licrocart 250-4 Licrosphere 100 Rp-18 (250 × 4 mm, 5 μm)	A: KDP 2.3 g L^{-1}: DSP 5.933 g L^{-1} in H_2O (pH 7.2), B: ACN	32 min/ 23 °C	Fl λex/em: 330/450 nm	–	–	[100]
	Wine	His, Tyr, Put, Cad, Met, Try, Eth, Ety; Others: Aa	–	–	1 min	C18 RP Waters AccQ.Tag, (3.9 × 150 mm, 4 μm)	A: 0.02 M P buffer (pH 7.3):MeOH: THF (91.5:8:0.5), B: 0.02 M P buffer (pH 7.3): MeOH (20:80)	70 min/ 35 °C	Fl λex/em: 340/425 nm	50 –350	–	[101]
OPA/MCE	Wine	His, Tyr, Put, Cad, Try, Phe, Spm, Spmd, Ser	–	–	99 s	Pinnacle II C-18 (150 × 3.9 mm, 5 μm)	A: 0.05 M NaAc (pH 6.6):THF (96:4), B: MeOH	25 min	Fl λex/em: 340/420 nm	–	–	[102]
	Cider and Wine	His, Tyr, Put, Cad, Phe, Met, Ety	–	–	–	Waters Nova-Pak C18 (150 × 3.9 mm, 4 μm)	A: 10 mM $NaHPO_4 \cdot 12H_2O$, B: 1% 2-octanol in ACN: 10 mM $NaHPO_4 \cdot 12H_2O$ (70:30)	60 min	Fl λex/em: 340/425 nm	–	–	[103,104]
	Wine	His, Tyr, Put, Cad, Try, Ety, Isoa	–	–	–	Spherisorb ODS2 (250 × 4.6 mm, 5 μm)	A: 0.05 M NaAc (pH 6.6):THF (99:1), B: MeOH:ACN (50:50)	33 min/ 45 °C	Fl λex/em: 340/420 nm	–	–	[105]
	Wine	His, Tyr, Put, Cad, Agm, Phe, Eth, Isoa, Isob, Try, Ser	–	–	1 min	Synergi-Hydro RP-80 Å (150 × 4.6 mm, 4 μm)	A: NaAc 0.05 M (pH 6.6):THF (99:1), B: MeOH:ACN (50:50)	46 min	Fl λex/em: 340/426 nm	15 –110	56 –117	[106]
	Wine and Beer	His; Others: Aa	0.1 M HCl	–	15 min/RT	Xtimate C18 (250 × 4.6 mm, 5 μm)	A: 25 mM NaAc buffer:THF (95:5), B: MeOH	60 min	Fl λex/em: 340/450 nm	0.235	102	[107]
OPA/NAC	Grape juice and Wine	His, Tyr, Put, Cad, Isoa, Phe, Eth; Others: Aa	–	–	3 min	Equisil® (250 × 3 mm, 5 μm)	A: 0.05 M NaAc buffer (pH 6.5): MeOH (95:5), B: MeOH:ACN (70:30)	39 min/ 25 °C	Fl λex/em: 330/440 nm	LOQ: 10-100	–	[24]
NITC	Fruits juices and beer	Tyr, Put, Try, Phe, Met, Spmd, Cys	TCA	SALLE (Derivatized BA extraction)	25 min/ 60°C	Princeton (250 × 4.6 mm, 5 μm)	ACN:H_2O (60:40)	20 min	UV–vis λ: 254 nm	0.06 –5.71	93 –110	[22]
PITC	Beer and Wine	Met, Dimet, Eth	Degassed	GDME	10 min/RT	Eurospher 100-5 C18 (250 × 4.0 mm, 5 μm)	A: ACN, B: H_2O/0.1% FA	25 min/ RT	UV–vis λ: 240 nm	12–46	–	[108]

UPLC												
DNS-Cl	Beer	His, Tyr, Put, Cad, Met, Phe, Spmd; Others: Aa	0.1 M HCl	–	60 min/40 °C	AcquityTM BEH C18 (100 × 2.1 mm, 1.7 μm)	A: H_2O/1% AcAc B: ACN/1% AcAc	30 min/ 40 °C	MS (ESI+)	5–20	104 –148	[109]
	Rice wine	His, Tyr, Put, Cad, Agm, Try, Phe, Spm, Spmd	5-sulfosalicylic acid	Ethyl acetate (Derivatized BA extraction)	60 min/50 °C	Zorbax Eclipse XDB-C18 (50 × 4.6 mm, 5 μm)	A: 20 mM AmAc (pH 3.5)/0.1% FA B: ACN/0.1% FA	21 min/ RT	MS/MS (ESI+)	0.1 –4.6	90 –109	[110]
	Bokbunja wines	His, Tyr, Put, Cad, Try, Phe, Spmd	–	SPE	45 min/40 °C	AcquityTM BEH C18 (50 × 2.1 mm, 1.7 μm)	A: H_2O/1% AcAc B: ACN/1% AcAc	13 min/ 40 °C	MS (ESI+)	3–15	71 –123	[111]
OPA/MCE (postcol)	Wine	His, Tyr, Put, Cad, Agm, Spm, Spmd, Try; Others: precursor Aa	–	–	40 °C	Prototype YMC-Triart C18 (3.0 × 50 mm, 2.2 μm)	A: 10 mM SDP and 150 mM SP (pH 2.0)/20 Mm PSAS, B: 10 mM SDP and 150 mM SP (pH 2):MeOH (50:50)/20 mM PSAS	8 min/ 40 °C	Fl λex/em: 345/455 nm	18.0 –78.8 fmol	98 –100	[112]
Ion-Pair LC												
NFPA	Beer and Vinegar	His, Tyr, Put, Cad, Try, Agm, Phe, Spm, Spmd, Ser, Dop, Oct; Others: β-ala, GABA	–	–	–	Luna Phenyl-Hexyl (150 × 2 mm, 3 μm)	A: H_2O/0.3% NFPA, B:MeOH/0.3% NFPA	45 min/ 20 °C	CLND	100 –400	98 –103	[50]
Chaotropic salt (KPF_6)	vinegar	His, Tyr, Try, Phe, Ser, Oct	–	0.1 M BEHPA in chloroform	–	Agilent Zorbax SB-C8 (150 × 4.6 mm, 5 μm)	A: 50 mM KPF_6 in 10 mM H_3PO_4 (pH 2.2), B: ACN	11 min/ 30°C	UV–vis λ: 210 nm	90 –150	90 –99	[113]
IC												
–	Fruits juice	His, Tyr, Put, Cad, Phe	Degassed	–	–	Metrosep C 4–100/4.0	5 mM nitric acid	40 min	CMD	56 –1630	87 –103	[33]
	Wine	His, Tyr, Put, Cad, Agm, Spm, Spmd, Tea, Tma	20 mM MSA	SPE	–	Dionex IonPac CS17 (250 × 4 mm, 7 μm)	MSA 6–40 mM	40 min/ RT	CMD	23-68 μg/kg	90 –99	[114]
TLC												
DNS-Cl	Wine	His, Tyr, Put, Cad	PVP	*Iso*-hexane	60 min/55 °C	Silicagel 60 TLC glass plates	Chloroform:Tea (4:1)	90 min	UV–vis λ: 312 nm	600 –700	89 –109	[115]
GC												
IBCF	Beer	18 BA	–	DLLME	9 min	DB-5MS capillary column, (20 m × 0.18 mm, 0.18 μm film thickness)	Helium	25 min/ 280 °C	MS	0.3 –2.9	72 –113	[48]
	Grape juice and wine	22 BA	–	Toluene	10 min	HP-5MS capillary column, (30 m × 0.25 mm, 0.25 μm film thickness)	Helium	25 min/ 280 °C	MS	1	–	[116]

Der.: Derivatization; BA: Biogenic amines; prep.: preparation; LOD: Limits of detection; Rec.: Recovery; Ref.: References; Aa: Amino acids; RT: Room temperature.
Analytes: His: Histamine; Tyr: Tyramine; Put: Putrescine; Cad: Cadaverine; Met: Methylamine; Agm: Agmatine; Phe: Phenylethylamine; Spm: Spermine; Spmd: Spermidine; Try: Tryptamine; Dimet: Dimethylamine; Hex: Hexylamine; Diety: Diethylamine; Ety: Ethylamine; Pyr: Pyrrolidine; Isob: Isobutylamine; Isop: Isopropylamine; Amy: Amylamine; Ser: Serotonin; Eth: Ethanolamine; Mpa: Methylpropylamine; 3-Mba: 3-Methylbutylamine; 2-Mba: 2-methylbutylamine; 3-Mtpa: 3-(Methylthio)propylamine; β-ala: β-alanine; Dap: Diaminopropane; Isoa: Isoamylamine; Oct: Octopamine; Isope: Isopentylamine; Dop: Dopamine; Cys: Cysteamine; GABA: γ-aminobutyric acid; Tea: Triethylamine; Tma: Trimethylamine.
Chemicals: PVP: polyvinylpyrrolidone; TCA: Trichloroacetic acid; DCM: Dichloromethane; PCA: Perchloric acid; MSA: Methanesulfonic acid; BEHPA: Bis (2-ethylhexyl) phosphate; FA: Formic acid; ACN: Acetonitrile; AF buffer: Ammonium formate buffer; PFHA: Perfluoroheptanoic acid; MeOH: Methanol; NaAc: Sodium acetate; AmAc: Ammonium acetate; Ac buffer: Acetate buffer; P buffer: Phosphate buffer; AcAc: Acetic acid; THF: Tetrahydrofuran; KDP: Potassium dihydrogenophosphate; DSP: Di-sodium hydrogenophosphate; SDP: Sodium dihydrogen phosphate; SP: Sodium perchlorate; PSAS: 1-Pentanesufonic acid sodium salt.
CMD: Conductometric detection.

[29] even though they used a UV–vis and DNS-Cl as a derivatization reagent (Table 2). This method was developed in order to determine BA in different food matrices, such as orange juice or ketchup (Table 2); and the authors suggested that this improvement was due to a large sample volume (50 μL) injected into the chromatograph.

o-Phthalaldehyde (OPA) has been often used in wines. Due to its instability, OPA is usually used together with other reagents such as mercaptoethanol (MCE) to improve the sensitivity and stability of derivatives [3]. Alternatively, Kelly et al. [24] validated a method to determine amino acids and some BA using OPA with *N*-acetyl-L-cysteine (NAC), which has shown to produce more stable derivatives. Numerous works that employ this reagent, avoiding the use of any samples pretreatment, have been recently published, this fact being surprising due to the low specificity of OPA [63]. Nevertheless, a method using UPLC and online post-column derivatization with OPA/MCE was able to reach very sensitive LODs (18.0–78.8 fmol) without sample pretreatment (Table 2).

Another derivatization reagent which is widely used is benzoyl chloride, as it has numerous advantages such as being relatively inexpensive, as well as the fact that its derivatives are stable [123]. Nevertheless, it is also a non-specific reagent, and it could react with other groups (phenols, aliphatic alcohols and some sugars), just like DNS-Cl does [117]. For these reasons, samples were often treated with PVP, hydrochloric acid or trichloroacetic acid (Table 2). On the other hand, methods with benzoyl chloride usually perform some type of liquid extraction after the derivatization reaction (Table 2). Benzoyl chloride has recently been used in different matrices, such as wine, beer, rice wine or şalgam (Table 2).

Regarding AQC, this derivatization reagent has shown to be specific and suitable for the determination of BA, since it produces compounds that remain stable for a week [3]. The excess reagent peak (6-aminoquinoline) could produce interferences when using a UV detector due to its high absorption, but its signal is smaller when detection is performed by fluorescence [122]. This derivatization reagent has recently been used in wine [69–71] and vinegar [49]. Ancín-Azpilicueta et al. [69] performed a chromatography method that determined 14 BA in 32 min. However, this method could not separate phenylethylamine and spermidine, which were quantified as a single peak. The employment of a SPE step prior to derivatization in vinegars allowed to clean up the samples and could improve their LODs (Table 2).

DEEMM is another reagent that produces stable derivatives and does not yield excess reagent [124]. The derivatization reaction is carried out for over 30 min in ultrasound bath, but it needs to heat at 70–80 °C for 2 h so as to allow the complete degradation of excess DEEMM. Several articles which use this reagent in wines and fermented products have recently been published [39,52,81,82]. The validated method [124] allowed the simultaneous determination of amino acids, BA and ammonium ion. Wang et al. [82] managed to reduce the time of analysis from 85 min to 30 min. However, these methods accounted for a lower sensitivity than others which used different derivatization reagents (Table 2).

CNBF reagent has numerous advantages, such as its fast reaction, the fact that it does not need large volumes of solvent and that its derivatives are photostable at room temperature. Nevertheless, this compound can react with other amino groups, and excess of CNBF could affect BA determination. This reagent was applied in two methods, involving different pretreatment techniques and derivatization reaction conditions (Table 2). Piasta et al. [79] aimed to prepare BA derivatives in high yield and purity, and they characterized pure CNBF derivatives by ^{1}H, ^{13}C, ^{19}F nuclear magnetic resonance (NMR), and single crystal X-ray crystallography, obtaining lower sample contamination with derivatization by-products.

To a lesser extent, other derivatization reagents, such as phenyl isothiocyanate (PITC) or 9-fluorenylmethyl chloroformate (FMOC), were applied in the past in fermented beverages samples [3,58]. Valente et al. [108] validated a method that simultaneously extracted (using GDME) and derivatized (using PITC) and, in which detection was performed by UV–vis, being the LODs for methylamine, dimethylamine and ethylamine were sensitive enough (12–46 μg L^{-1}). However, the most important drawback is that the main BA were not determined by this method. In addition, a method for wine and beer which applied VSLLME and FMOC as reagent was validated. In this case, the extraction was relevant because this derivatization reagent reacts with some products which can interfere in the analysis. This method could determine five BA, obtaining satisfactory LODs ranging 1–2.6 μg L^{-1} (Table 2), but it was not able to detect putrescine [99].

On the other hand, new derivatization reagents are being implemented, showing new advantages such as good selectivity and sensitivity. Among them, we can find 2, 6-dimethyl-4-quinolinecarboxylic acid *N*-hydroxysuccinimide ester (DMQC-OSu), ethyl-acridine-sulfonyl chloride (EAC) or 1-naphthylisothiocyanate (NITC) [22,83,98].

In a recent research in beer samples, the fluorescence labeling reagent DMQC-OSu was employed. The method showed a good selectivity and provided few by-products [83]. However, this chromatographic method only determined tyramine, octopamine and phenylethylamine, which are commonly present in beer at low concentrations (Table 2). On the other hand, Li et al. [98] developed and validated a method that used EAC as fluorescence labeling reagent to determine BA and estrogens in several food samples, obtaining good LODs (0.27–0.67 μg L^{-1}) (Table 2).

Isotope-coded derivatization reagents d_0-10-methyl-acridone-2-sulfonyl chloride (d_0-MASC) and d_3-10-methyl-acridone-2-sulfonyl chloride (d_3-MASC) were used to determine seven BA in Chinese rice wine samples. A fast LC method (5 min) was used, obtaining good LODs (Table 2). However, the main disadvantages of this method were that the derivatization reagent needs a laborious preparation and that it is expensive [80].

On the other hand, a method that employed ion-pair liquid chromatography using perfluorocarboxylic acids as ion-pair reagents and chemiluminescent nitrogen detector (CLND) was validated and optimized [50]. Nonafluoropentanoic acid (NFPA) was selected as the best ion-pair reagent among the several perfluorocarboxylic acids tested. Alternatively, another method of ion-pair liquid chromatography used chaotropic salt KPF_6 and was applied in vinegar [113]. However, the LODs in both methods did not improve in comparison with those methods that used the derivatization reagents (Table 2).

Although a previous derivatization is usually needed to simultaneously determine BA, some recent articles aimed to avoid the use of reagents by exclusively detecting histamine. Hence, a new method that used aqueous normal phase (ANP) conditions and silica hydride-based stationary phase was applied by using LC with UV–vis and MS detection, thus avoiding longer analysis times and increased impurities due to derivatization [65]. The calculated LOQ value did not improve in comparison with other methods which used a derivatization reagent and the same detector (Table 2). On the other hand, a method performed by Cai et al. [67] using nano extractive electrospray ionization mass spectrometry (nano EESI-MS) obtained a lower LOD (20 μg L^{-1}) for histamine in beer samples (Table 2).

2.1.3. Analytical determination

The most common methods used to determine BA usually employed LC with C18 column, as mentioned above. Regarding the time of analysis, it was widely variable and it mainly depended on

the number of analytes under consideration, ranging between 5 and 85 min (Table 2). Those methods that involved the simultaneous determination of amino acids and BA lasted, in all cases, more than 30 min. On the other hand, more recently, BA determination has been performed by UPLC. This technique offers many advantages over LC, particularly, reducing run time and solvent consumption [110,111]. A Kinetex core-shell PFP LC column was recently used as an alternative to C18 column. This method employed AQC, and the authors concluded that Kinetex PFP column mainly reduced the run times (25 min from 50 min) and solvent volumes (20 mL from 47 mL per sample) [70]. However, their LOQs were higher than in other methods (Table 2).

Detection by UV–vis and fluorescence has usually been employed in most cases. LC-MS has recently been applied to increase the reliability of BA identification [25]. On the other hand, BA could be detected directly by using positive ionization [125].

In general, those methods using fluorescence detection were more sensitive than UV–vis, regardless of the reagent employed (Table 2). Although MS detection has also been employed, the LODs obtained were not significantly lower than those reached by fluorescence detection, and this fact explains its widespread use (Table 2). Also noteworthy was a new method recently performed by Mayr and Schieberle [25], that involved the use of isotopically labeled internal standard for 14 BA and two polyamines, obtaining low LOQ (around 0.05 μg kg^{-1}) and employing tandem mass spectrometry (MS/MS) detector. MS/MS is able to provide specific structural information and a more sensitive detection capability [110]. Besides, isotopically labeled internal standards have been proved to minimize matrix interferences in complex matrices [25,80]. Thus, an ultratrace analysis was accomplished.

Alternatively, a couple of methods used ion chromatography to determine BA in juice fruits and wines [33,114]. Although the LODs obtained were at the same order of magnitude than RP-LC (Table 2), these methods had the advantage that they did not need any derivatization reagent.

Thin-layer chromatography (TLC) could represent an alternative to previous methods with LC. This technique had different advantages; for example, it does not need a special equipment and it could analyze several samples at the same time [115,121]. Romano et al. [115] recently employed this technique to determine the main BA in wine samples, which were treated with PVP to avoid interferences and were derivatized with DNS-Cl (Table 2). The main disadvantages were that the analyses time is 90 min, as well as the fact that this method is semi-quantitative. On the other hand, the LODs obtained with this method were not very sensitive (600–700 μg L^{-1}).

2.2. Gas chromatography

Gas chromatography methods are not so often applied to determine biogenic amines in food matrices. It has been specifically used in fermented beverages by some authors in order to offer alternative methods to LC [120]. It needs a previous derivatization step in order to increase volatile properties and to decrease polarities of BA [120]. Two GC-MS methods were recently developed and validated for the determination of biogenic amines in beers [48] and wines (Port wines) [116], using isobutyl chloroformate (IBCF) as derivatizing agent. In the first method, the derivatization procedure was simultaneously performed with a dispersive liquid-liquid microextraction (DLLME), whilst in the second one, derivatization was carried out in a two-phase reaction system, eliminating the need for a previous extraction procedure. Both methods showed to be efficient and highly reproducible, allowing the accurate identification and quantification of a higher number of biogenic amines than LC methods (Table 2). In addition, the derivatization procedure and the overall time of analysis were faster than in some LC methods, reaching LODs of the same order than them (Table 2).

2.3. Capillary electrophoresis

CE methods can currently be divided into the following ones: capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), capillary isotachophoresis (CITP), capillary isoelectric focusing (CIEF) and micellar electrokinetic chromatography (MEKC) [126]. CE can be considered as a good option for the determination of biogenic amines in foods [57], despite its low sensitivity when compared with other detection methods. This technique is fast and efficient, as well as inexpensive in terms of reagents consumption, and it shows a lower reproducibility of migration times than LC [127]. Hence, its suitability will depend on the expected BA levels in fermented beverages samples. Since the maximum levels for histamine are legally established for fish products, most of the applications using CE are related to these products [128,129]. Off-line precolumn derivatization is the most common application; the resulting derivatives are injected into the CE and in the case of wines, the detection limits reached in these methods are fully compatible with the typical levels of BA. Furthermore, the latest advances in derivatization strategies and some innovative on-line pre-concentration procedures have contributed to enhance the sensitivity of methods. A previous step involving clean-up, such as added PVP followed by filtration, is usually performed [130].

Table 3 summarizes the latest developments in wine and beer analysis. A CE-MS/MS method has been used for the quantitative determination of BAs in beer and wine samples [130]. This method is fast due to the short migration time for the 9 BAs (spermine, spermidine, putrescine, cadaverine, histamine, phenylethylamine, tryptamine, tyramine, and urocanic acid) (<10 min) and the enhanced sensitivity, since the LODs and LOQs were in the range of 1–2 μg L^{-1} and 3–8 μg L^{-1} for wine and beer, respectively. The use of a PVA-coated silica capillary allows to suppress the electroosmotic flow and, as a consequence, to increase the separation efficiency [130]. The online sample preconcentration and online combined CZE with CITP, coupled to photometric detection at 280 nm, can also improve the sensitivity [127]. The LODs were 0.35 mg L^{-1} for histamine, 0.33 mg L^{-1} for phenylethylamine and 0.37 mg L^{-1} for tyramine. This method has been successfully employed for the determination of histamine, 2-phenylethylamine and tyramine in red wine samples (Table 3). Jastrzębska et al. [132] performed a comparative study between a CITP method without a derivatization step and a LC method using DNS-Cl as derivatization reagent and detection by UV–vis. This CITP method obtained better detection limits than the LC method with UV detector.

Uzasci et al. [133] reported a novel nonionic micellar electrokinetic chromatography method through fluorescein isothiocyanate isomer I (FITC) coupled to laser-induced fluorescence detector (LIF). They used Brij 35 instead of SDS as surfactant, thus decreasing the separation time and obtaining a good sensitivity. A fast separation of seven biogenic amines was performed and LODs were between 0.42 and 1.26 nM. This method was applied to determine biogenic amines in wine samples and fruit molasses.

2.4. Others

Alternatively to chromatography, other techniques such as sensors, ELISA and flow-injection analysis have been employed to determine biogenic amines in fermented beverages.

Sensors are an interesting alternative to LC methods, since they do not require a special instrumentation, and do not need sample clean-up and derivatization procedures, thus being in general less-

Table 3
Biogenic amine determination by capillary electrophoresis.

Electrophoretic method	Matrix	BA and other compounds	Sample preparation	Separation techniques	Run time	Detection	LOD (μg L^{-1})	Rec. (%)	Ref.
Microchip capillary electrophoresis	Dark beer	Eth, Try; Other: Tryptophan	Degassed and P buffer	Glass microchannel chips, model MC-BF4-001 Running buffer: 20 mM phosphate (pH 2.5) and a high voltage of 2.5 kV	4 s	Electrochemical (Ruthenium-containing films coated glassycarbon electrodes)	1400–6800	–	[131]
Capillary isotachophoresis (CITP)	Beer and Wine	His, Tyr, Put, Cad, Phe, Try, Spm, Spmd	Degassed (Beer)	PTFE analytical capillary 160 mm, 300 μm; Leading electrolyte: 5 mM $Ba(OH)_2$ + 15 mM valine + 1% hydroxyethylcellulose (pH 8.5), terminating electrolyte: 0.02 M TRIS + 0.1 M HCl (pH 8.3).	14 min	Conductometric detector	200–480	90–101	[132]
Non-Ionic Micellar Electrokinetic Chromatography (MECK)	Wine	His, Tyr, Put, Cad, Spmd, Try, Phe	Derivatization with FITC	Silica capillary 580 mm, 50 μm Running buffer: Brij 35, borate buffer (pH 9.6)	9 min/ 25 °C	LIF λex/em: 488/520 nm	0.06–0.11	93–104	[133]
Capillary isotachophoresis-Capillary zone electrophoresis (CITP-CZE)	Wine	His, Tyr, Phe	–	CITP: column of 140 mm, 800 μm; CZE: column of 160 mm, 300 μm Separation electrolyte: CITP: Leading electrolyte: K (pH 6), terminating electrolyte: EACA (pH 4.3). CZE: Background electrolyte: GABA (pH 4.1)	35 min	UV λ: 280 nm	330–370	92–96	[127]
Capillary electrophoresis	Beer and wine	His, Tyr, Put, Cad, Spm, Spmd, Try, Phe, Uro	Degassed and PVP	PVA-coated silica capillary 700 mm, 50 μm Separation electrolyte: Background electrolyte: 0.5 M acetic acid (pH 2.5)	10 min	MS/MS (ESI+)	1–2	87–113	[130]

BA: Biogenic amines; LOD: Limits of detection; Rec.: Recovery; Ref.: References; FITC: Fluorescein isothiocyanate isomer I; LIF: Laser-induced fluorescence detector. PTFE: polytetrafluoroethylene.
Analytes: His: Histamine; Tyr: Tyramine; Put: Putrescine; Cad: Cadaverine; Phe: Phenylethylamine; Spm: Spermine; Spmd: Spermidine; Try: Tryptamine; Eth: Ethanolamine; Uro: Urocanic acid.
Chemical: PVP: polyvinylpyrrolidone; P buffer: Phosphate buffer.

time consuming. Therefore, sensors are an interesting approach due to their short analysis time, low cost and simplicity. Besides, an important advantage of this technique is that it may be used for *in situ* analysis [121].

Sensors have been applied for wine and beer, requiring just a pH adjustment (Table 4). Most methods were able to detect just a concrete amine or an overall total amount of BA in fermented beverages samples (Table 4). Exceptionally, a dual enzymatic sensor was developed to simultaneously determine histamine and putrescine [139]. On the other hand, there is a wide variety of recognition elements [141], being enzymatic biosensors the most prevalent for BA determination (Table 4). Alternatively, Bazosabal et al. [134] developed a sensor with molecularly imprinted nanoparticles which had a high affinity for histamine.

Regarding transduction systems, electrochemical and optical sensors have been employed to detect BA. As for electrochemical sensors, the methods that employed potentiometric sensors were most sensitive than those methods with amperometric determination (Table 4). Alternatively, Ramon-Marquez et al. [138] developed a novel optical sensor in combination with nanotechnology. This sensor detected tryptamine by using the intrinsic phosphorescence property. Hence, it was covalent immobilized in a nanofiber mat and quantified by phosphorescence (solid surface-room temperature phosphorescence), which was highly sensitive in beer (6 μg L^{-1}).

On the other hand, ELISA (Enzyme-Linked Immuno Sorbent Assay) test is a technique with a high precision, sensitivity and good potential for standardization [142]. It has been applied for the determination of histamine in fish and, more recently, in other matrices such as wine [143]. However, it can be time-consuming and expensive, in particular for a small number of samples [142]. Recently, Luo et al. [144] developed a hapten that employed

Table 4
Biogenic amine determination by sensors.

Matrix	BA and other compounds	Sample preparation	Recognition element	Type of chemical sensor or biosensor (principle of transduction)	Temperature	LOD (μg L^{-1})	Rec. (%)	Ref.
Wine	His	5 NaOH and 10 mM Ac buffer (pH 5)	Molecularly imprinted nanoparticles (MIN)	Electrochemical sensor (potentiometry)	RT	124	99–102	[134]
Beer	Put	Degassed	Putrescine oxidase	Electrochemical sensor (amperometry)	–	440.8	85	[135]
Beer	Tyr	–	Tyramine oxidase	Electrochemical sensor (amperometry)	35 °C	1371.8	96–97	[136]
Beer	Tyr	–	Tyramine oxidase	Electrochemical sensor (amperometry)	35 °C	2400	96–97	[137]
Beer	Try	Degassed and 20 mM carbonate buffer (pH 10)	Solid phase (Michael type-reaction)	Optical sensor (phosphorescence) λex/em: 290/443 nm	RT	6	86–116	[138]
Wine	His, Put	50 mg activated carbon	Putrescine oxidase and Histamine deshydrogenase	Electrochemical sensor (amperometry)	RT	881.5–900.3	–	[139]
Beer and Wine	Total BA	0.1 M phosphate and NaOH (pH 7.4)	Diamine oxidase	Electrochemical sensor (amperometry)	35 °C	200	–	[140]

BA: Biogenic amines; LOD: Limits of detection; Rec.: Recovery; Ref.: References; RT: Room temperature.
Analytes: His: Histamine; Tyr: Tyramine; Put: Putrescine; Try: Tryptamine.
Chemical: Ac buffer: Acetate buffer.

antibodies against histamine that had previously been derivatized with *p*-nitrobenzoic acid *N*-hydroxysuccinimide ester (PNBA-OSu). The derivatization reaction was performed at room temperature for 20 min. This derivative had a better binding affinity and specificity with antibodies than free histamine. Moreover, this test had a good correlation ($R^2 = 0.978$) with LC–MS/MS. Thus, recovery and LOD were suitable for determining histamine in wines with a low concentration (93.6 μg L^{-1}), and they were similar to those in LC methods (Table 2).

Another alternative is the flow-injection analysis. Traditionally, this technique consists in the injection of sample into a carrier solution that is in a constant flow, where both solutions are mixed and passed through a detector [145]. Recently, Hernández-Cassou and Saurina [145] employed this technique to determine histamine in commercial red wines. The researchers used the reagent 1,2-naphthoquinone-4-sulfonate to improve the detection of this BA by UV–Vis. Besides, a multivariate calibration method (Partial least square) was used to overcome the interference of amino acids or polyphenols, improving the selectivity. Thus, this method had a good correlation ($R^2 > 0.99$) with LC method and the analysis was fast (residence time of 0.76 min). However, this method was optimized just for histamine.

3. Applicability in food samples

The interest of BA determination in food and beverages has to do with food safety, production process monitoring or food microbiology research.

Histamine, tyramine, putrescine and cadaverine are the main BA which are commonly detected in fermented beverages, mainly wine and beer (Table 1) [62]. Their final contents in wine are related to a number of variables such as vintage, grape variety, levels of amine-precursor amino acids, and above all winemaking practices. Thus, even though BA are present after alcoholic fermentation, they are mainly produced during malolactic fermentation in red wines [71,146]. Therefore, the expected concentrations of these BA in wines are in the range of mg L^{-1}.

In general, BA concentrations in beer are lower than in wine (Table 1). Although small quantities of BA are formed during alcoholic fermentation, they could increase significantly if beers are contaminated with lactic acid bacteria (LAB) during the brewing process [147] or during their storage [148]. Hence, BA concentrations in properly stored beer hardly reach some mg L^{-1}.

Other fermented products, such as vinegars and gluconic fermented beverages, showed very low concentrations of BA; in fact, acetic acid bacteria have been reported as no BA producers [49,149].

Regarding food safety, researches were mainly focused on final products, as they are the ones which reach the market, as well as on storage control [49,84,91,106]. Moreover, most new developed methods were adapted and validated using final products in order to verify their application in one or several matrices [50,91,98,99,109,114]. Hence, a number of studies usually compared BA concentrations in different types of wine [44,90], grape varieties [41,88], agricultural practices (Organic and non-organic) [40], ageing period [101], and controlled designation of origin (DO) and authentication [43,94]. Besides, levels of BA are used as indicators of quality and safety of wines [44]. Regarding other fermented products, BA have been determined in different types of beers [48,73,84,98] and vinegars [49].

BA have been also considered in metabolomic studies. Thus, the metabolome profile during the malting process of barley based on GC-MS without derivatization [150] was studied. Among the hydrophilic barley compounds identified (n = 65), those BA having a low molecular weight, such as putrescine and ethanolamine, were detected, and their concentration increased in the course of malting, mainly in the case of putrescine. Arbulu et al. [151] performed an untargeted metabolomic method for the non-volatile profile of Graciano wine with LC-MS, which determined that 4% of the total number of metabolites identified were BA. Moreover, they elaborated an enological database (WinMet), which is a useful tool for wine metabolomic characterization and authenticity testing [151].

Storage conditions and their impact on BA concentrations have been also considered within wine production [106,152]. Thus, Ceccini et al. [7] monitored the effect of the grape storage time in the final content of BA in must. However, storage control studies have been mainly focused on final products which were kept in

barrels or bottles [106,152].

Production process monitoring is important in order to control the synthesis or degradation of BA. As mentioned above, sensitive methods are necessary to be able to observe significant changes. Thus, changes in winemaking process have been widely studied taking into account grape variety, vintage, DO … [71,81,82,106]; and the same applies to cider and rice wine, from harvest to bottling [87,103].

The interests of food microbiology research have been mainly focused on the molecular mechanisms that control the synthesis of BA, as well as the evaluation of the BA-degrading capacity of microorganisms. Thus, several LAB species, isolated from fermented beverages, proved to be able to degrade biogenic amines in wine through the production of amine oxidase enzymes [104]. Some species showed a good amine-degrading ability; however, their activity was limited by ethanol, polyphenols or SO_2. Hence, the authors concluded that their applicability in winemaking still needs further investigation.

The current challenges facing the BA research are: reducing the time of analysis, being able to use a lower concentration of derivatization reagent and increasing sensitivity. Therefore, in the context of future regulations of BA in fermented products, fast and robust methods of analysis will be required.

As aforementioned, the determination of BA concentrations below mg L^{-1} was not necessary regarding food safety and health concerns. Thus, the current legislation in fishery products, as well as those recommendations established for histamine in wine and beer, had a threshold of several mg L^{-1}. Hence, LC methods coupled with UV–vis or fluorescent detector are adequate to determine BA in the laboratory, not being necessary to use a MS detector.

Regarding quality control and research requirements, sensitive methods, as well as a more precise identification of analytes, are required to detect slight changes in BA profile. Thus, MS or MS/MS detectors are the most adequate so as to detect low concentrations of metabolites, due to their sensitivity and the specific structural information that they provide. On the other hand, the matrix effect remains one of the main problems when quantifying BA, especially in food samples which are composed by complex matrices. Thus, the sample preparation step has been commonly used so as to avoid matrix interferences [153]. However, the isotopically labeled internal standards are the most reliable method to minimize the matrix effect, allowing the quantification of ultratraces [25].

Acknowledgements

We thank the Ministry of Economy and Competitiveness of the Spanish government for its financial support of project AGL2013–47300–C3–2–R and the “Consejería de Economía, Innovación y Ciencia, Junta de Andalucía” under the project P12-AGR-1601.

References

[1] EFSA Panel on Biological Hazards (BIOHAZ), Scientific opinion on risk based control of biogenic amine formation in fermented foods, EFSA J. 9 (2011) 93.
[2] G. Spano, P. Russo, A. Lonvaud-Funel, P. Lucas, H. Alexandre, C. Grandvalet, et al., Biogenic amines in fermented foods, Eur. J. Clin. Nutr. 64 (2010) 95–100.
[3] A. Peña-Gallego, P. Hernández-Orte, J. Cacho, V. Ferreira, High-performance liquid chromatography analysis of amines in must and wine: a review, Food Rev. Int. 28 (2012) 71–96.
[4] E. Peñas, J. Frias, B. Sidro, C. Vidal-Valverde, Impact of fermentation conditions and refrigerated storage on microbial quality and biogenic amine content of sauerkraut, Food Chem. 123 (2010) 143–150.
[5] N. Toro-Funes, J. Bosch-Fuste, M.L. Latorre-Moratalla, M.T. Veciana-Nogués, M.C. Vidal-Carou, Biologically active amines in fermented and non-fermented commercial soybean products from the Spanish market, Food Chem. 173 (2015) 1119–1124.
[6] B.Y. Byun, X. Bai, J.H. Mah, Occurrence of biogenic amines in doubanjiang and Tofu, Food Sci. Biotechnol. 22 (2013) 55–62.
[7] F. Cecchini, M. Morassut, Effect of grape storage time on biogenic amines content in must, Food Chem. 123 (2010) 263–268.
[8] M. Saaid, B. Saad, N.H. Hashim, A.S. Mohamed Ali, M.I. Saleh, Determination of biogenic amines in selected Malaysian food, Food Chem. 113 (2009) 1356–1362.
[9] A. Halász, Á. Baráth, W.H. Holzapfel, The biogenic amine content of beer; the effect of barley, malting and brewing on amine concentration, Z. Leb. Unters. Forsch. A 208 (1999) 418–423.
[10] J.S. Min, S.O. Lee, a. Jang, C. Jo, C.S. Park, M. Lee, Relationship between the concentration of biogenic amines and volatile basic nitrogen in fresh beef, pork, and chicken meat, Asian-Aust. J. Anim. Sci. 20 (2007) 1278–1284.
[11] V. Sirocchi, G. Caprioli, M. Ricciutelli, S. Vittori, G. Sagratini, Simultaneous determination of ten underivatized biogenic amines in meat by liquid chromatography-tandem mass spectrometry (HPLC-MS/MS), J. Mass Spectrom. 49 (2014) 819–825.
[12] C.A. Lázaro, C.A. Conte-Júnior, A.C. Canto, M.L.G. Monteiro, B. Costa-Lima, A.G. Da Cruz, et al., Biogenic amines as bacterial quality indicators in different poultry meat species, LWT - Food Sci. Technol. 60 (2015) 15–21.
[13] R. Romero-González, M.I. Alarcón-Flores, J.L. Martínez Vidal, A. Garrido Frenich, Simultaneous determination of four biogenic and three volatile amines in anchovy by ultra-high-performance liquid chromatography coupled to tandem mass spectrometry, J. Agric. Food Chem. 60 (2012) 5324–5329.
[14] A. Dehaut, C. Himber, V. Mulak, T. Grard, F. Krzewinski, B. Le Fur, et al., Evolution of volatile compounds and biogenic amines throughout the shelf life of marinated and salted anchovies (*Engraulis encrasicolus*), J. Agric. Food Chem. 62 (2014) 8014–8022.
[15] H. Zhai, X. Yang, L. Li, G. Xia, J. Cen, H. Huang, S. Hao, Biogenic amines in commercial fish and fish products sold in southern China, Food Control 25 (2012) 303–308.
[16] B. Bilgin, H. Genççelep, Determination of biogenic amines in fish products, Food Sci. Biotechnol. 24 (2015) 1907–1913.
[17] Y. Hu, Z. Huang, J. Li, H. Yang, Concentrations of biogenic amines in fish, squid and octopus and their changes during storage, Food Chem. 135 (2012) 2604–2611.
[18] D.M. Linares, M. Cruz Martín, V. Ladero, M.A. Álvarez, M. Fernández, Biogenic amines in dairy products, Crit. Rev. Food Sci. 517 (2011) 691–703.
[19] D.M. Linares, B. Del Río, V. Ladero, N. Martínez, M. Fernández, M. Cruz Martín, M.A. Álvarez, Factors influencing biogenic amines accumulation in dairy products, Front. Microbiol. 3 (2012) 1–10.
[20] Ö. Özdestan, Evaluation of bioactive amine and mineral levels in Turkish coffee, Food Res. Int. 61 (2014) 167–175.
[21] D. Restuccia, U.G. Spizzirri, O.I. Parisi, G. Cirillo, N. Picci, Brewing effect on levels of biogenic amines in different coffee samples as determined by LC-UV, Food Chem. 175 (2015) 143–150.
[22] A. Jain, M. Gupta, K.K. Verma, Salting-out assisted liquid-liquid extraction for the determination of biogenic amines in fruit juices and alcoholic beverages after derivatization with 1-naphthylisothiocyanate and high performance liquid chromatography, J. Chromatogr. A 1422 (2015) 60–72.
[23] C. Basheer, W. Wong, A. Makahleh, A.A. Tameem, A. Salhin, B. Saad, H.K. Lee, Hydrazone-based ligands for micro-solid phase extraction-high performance liquid chromatographic determination of biogenic amines in orange juice, J. Chromatogr. A 1218 (2011) 4332–4339.
[24] M.T. Kelly, A. Blaise, M. Larroque, Rapid automated high performance liquid chromatography method for simultaneous determination of amino acids and biogenic amines in wine, fruit and honey, J. Chromatogr. A 1217 (2010) 7385–7392.
[25] C.M. Mayr, P. Schieberle, Development of stable isotope dilution assays for the simultaneous quantitation of biogenic amines and polyamines in foods by LC-MS/MS, J. Agric. Food Chem. 60 (2012) 3026–3032.
[26] G.G. Mohamed, A.K.A. El-Hameed, A.M.M.N. El-Din, L.A.M.M.N. El-Din, High performance liquid chromatography, thin layer chromatography and spectrophotometric studies on the removal of biogenic amines from some Egyptian foods using organic, inorganic and natural compounds, J. Toxicol. Sci. 35 (2010) 175–187.
[27] R.F. Guan, Z.F. Liu, J.J. Zhang, Y.X. Wei, S. Wahab, D.H. Liu, X.Q. Ye, Investigation of biogenic amines in sufu (furu): a Chinese traditional fermented soybean food product, Food Control 31 (2013) 345–352.
[28] X. Gong, N. Qi, X. Wang, L. Lin, J. Li, Ultra-performance convergence chromatography (UPC2) method for the analysis of biogenic amines in fermented foods, Food Chem. 162 (2014) 172–175.
[29] A.A. Tameem, B. Saad, A. Makahleh, A. Salhin, M.I. Saleh, A 4-hydroxy-N-[(E)-(2-hydroxyphenyl)methylidene]benzohydrazide-based sorbent material for the extraction-HPLC determination of biogenic amines in food samples, Talanta 82 (2010) 1385–1391.
[30] C. Ruiz-Capillas, M. Triki, A.M. Herrero, F. Jiménez-Colmenero, Biogenic amines in low- and reduced-fat dry fermented sausages formulated with Konjac Gel, J. Agric. Food Chem. 60 (2012) 9242–9248.
[31] E.J. Papavergou, I.N. Savvaidis, I. a. Ambrosiadis, Levels of biogenic amines in retail market fermented meat products, Food Chem. 135 (2012) 2750–2755.
[32] E. De Mey, G. Drabik-Markiewicz, H. De Maere, M.C. Peeters, G. Derdelinckx, H. Paelinck, T. Kowalska, Dabsyl derivatisation as an alternative for

dansylation in the detection of biogenic amines in fermented meat products by reversed phase high performance liquid chromatography, Food Chem. 130 (2012) 1017–1023.
[33] A. Jastrzębska, A. Piasta, E. Szłyk, Application of ion chromatography for the determination of biogenic amines in food samples, J. Anal. Chem. 70 (2015) 1131–1138.
[34] R. Stute, K. Petridis, H. Steinhart, G. Biernoth, Biogenic amines in fish and soy sauces, Eur. Food Res. Technol. 215 (2002) 101–107.
[35] L. Buňková, G. Adamcová, K. Hudcová, H. Velichová, V. Pachlová, E. Lorencová, et al., Monitoring of biogenic amines in cheeses manufactured at small-scale farms and in fermented dairy products in the Czech Republic, Food Chem. 141 (2013) 548–551.
[36] B. Redruello, V. Ladero, I. Cuesta, J.R. Álvarez-Buylla, M.C. Martín, M. Fernández, M.A. Álvarez, A fast, reliable, ultra high performance liquid chromatography method for the simultaneous determination of amino acids, biogenic amines and ammonium ions in cheese, using diethyl ethoxymethylenemalonate as a derivatising agent, Food Chem. 139 (2013) 1029–1035.
[37] S. Novella-Rodríguez, M.T. Veciana-Nogués, M. Izquierdo-Pulido, M.C. Vidal-Carou, Distribution of biogenic amines and polyamines in cheese, J. Food Sci. 68 (2003) 750–755.
[38] Ö. Özdestan, A. Üren, Biogenic amine content of kefir: a fermented dairy product, Eur. Food Res. Technol. 231 (2010) 101–107.
[39] B. Bach, S. Le Quere, P. Vuchot, M. Grinbaum, L. Barnavon, Validation of a method for the analysis of biogenic amines: histamine instability during wine sample storage, Anal. Chim. Acta 732 (2012) 114–119.
[40] P. Comuzzo, D. Rauhut, M. Werner, C. Lagazio, R. Zironi, A survey on wines from organic viticulture from different European countries, Food Control 34 (2013) 274–282.
[41] V. Konakovsky, M. Focke, K. Hoffmann-Sommergruber, R. Schmid, O. Scheiner, P. Moser, R. Jarisch, W. Hemmer, Levels of histamine and other biogenic amines in high-quality red wines, Food Addit. Contam. A 28 (2011) 408–416.
[42] M. Ortega-Heras, S. Pérez-Magariño, V. Del-Villar-Garrachón, C. González-Huerta, L.C. Moro Gonzalez, A. Guadarrama Rodríguez, S. Villanueva Sanchez, R. Gallo González, S. Martín de la Helguera, Study of the effect of vintage, maturity degree, and irrigation on the amino acid and biogenic amine content of a white wine from the Verdejo variety, J. Sci. Food Agric. 94 (2014) 2073–2082.
[43] C.I.G. Tuberoso, F. Congiu, G. Serreli, S. Mameli, Determination of dansylated amino acids and biogenic amines in Cannonau and Vermentino wines by HPLC-FLD, Food Chem. 175 (2015) 29–35.
[44] M. Martuscelli, G. Arfelli, A.C. Manetta, G. Suzzi, Biogenic amines content as a measure of the quality of wines of Abruzzo (Italy), Food Chem. 140 (2013) 590–597.
[45] X. Guo, X. Guan, Y. Wang, L. Li, D. Wu, Y. Chen, H. Pei, D. Xiao, Reduction of biogenic amines production by eliminating the PEP4 gene in Saccharomyces cerevisiae during fermentation of Chinese rice wine, Food Chem. 178 (2015) 208–211.
[46] S. Jia, Y. Ryu, S.W. Kwon, J. Lee, An in situ benzoylation-dispersive liquid-liquid microextraction method based on solidification of floating organic droplets for determination of biogenic amines by liquid chromatography-ultraviolet analysis, J. Chromatogr. A 1282 (2013) 1–10.
[47] E. Mazzucco, F. Gosetti, M. Bobba, E. Marengo, E. Robotti, M.C. Gennaro, High-performance liquid chromatography-ultraviolet detection method for the simultaneous determination of typical biogenic amines and precursor amino acids. applications in food chemistry, J. Agric. Food Chem. 58 (2010) 127–134.
[48] C. Almeida, J.O. Fernandes, S.C. Cunha, A novel dispersive liquid-liquid microextraction (DLLME) gas chromatography-mass spectrometry (GC-MS) method for the determination of eighteen biogenic amines in beer, Food Control 25 (2012) 380–388.
[49] J.L. Ordóñez, R.M. Callejón, M.L. Morales, M.C. García-Parrilla, A survey of biogenic amines in vinegars, Food Chem. 141 (2013) 2713–2719.
[50] J. Sun, H.-X. Guo, D. Semin, J. Cheetham, Direct separation and detection of biogenic amines by ion-pair liquid chromatography with chemiluminescent nitrogen detector, J. Chromatogr. A 1218 (2011) 4689–4697.
[51] G. Garai, M.T. Dueñas, a Irastorza, P.J. Martín-Alvarez, M.V. Moreno-Arribas, Biogenic amines in natural ciders, J. Food Prot. 69 (2006) 3006–3012.
[52] J.L. Ordóñez, F. Sainz, R.M. Callejón, A.M. Troncoso, M.J. Torija, M.C. García-Parrilla, Impact of gluconic fermentation of strawberry using acetic acid bacteria on amino acids and biogenic amines profile, Food Chem. 178 (2015) 221–228.
[53] M.B.H. Youdim, D. Edmondson, K.F. Tipton, The therapeutic potential of monoamine oxidase inhibitors, Nat. Rev. Neurosci. 7 (2006) 295–309.
[54] V. Ladero, M. Calles-Enriquez, M. Fernandez, M.A. Alvarez, Toxicological effects of dietary biogenic amines, Curr. Nutr. Food Sci. 6 (2010) 145–156.
[55] D.M. Linares, B. del Rio, B. Redruello, V. Ladero, M.C. Martin, M. Fernandez, P. Ruas-Madiedo, M.A. Álvarez, Comparative analysis of the in vitro cytotoxicity of the dietary biogenic amines tyramine and histamine, Food Chem. 197 (2016) 658–663.
[56] M.K. Kim, J.H. Mah, H.J. Hwang, Biogenic amine formation and bacterial contribution in fish, squid and shellfish, Food Chem. 116 (2009) 87–95.
[57] F. Bedia Erim, Recent analytical approaches to the analysis of biogenic amines in food samples, TrAC- Trend. Anal. Chem. 52 (2013) 239–247.
[58] T. Bauza, A. Blaise, F. Daumas, J.C. Cabanis, Determination of biogenic amines and their precursor amino acids in wines of the Vallé du Rhone by high-performance liquid chromatography with precolumn derivatization and fluorimetric detection, J. Chromatogr. A 707 (1995) 373–379.
[59] J.M. Landete, S. Ferrer, L. Polo, I. Pardo, Biogenic amines in wines from three Spanish regions, J. Agric. Food Chem. 53 (2005) 1119–1124.
[60] International Organisation of Vine and Wine (OIV), http://www.oiv.int/en/ (accessed March 31, 2016).
[61] J. Karovicova, Z. Kohajdova, Biogenic amines in food, Chem. Pap. 36 (2005) 479–484.
[62] P. Hernández-Orte, A. Peña-Gallego, M.J. Ibarz, J. Cacho, V. Ferreira, Determination of the biogenic amines in musts and wines before and after malolactic fermentation using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate as the derivatizing agent, J. Chromatogr. A 1129 (2006) 160–164.
[63] A. Peña-Gallego, P. Hernández-Orte, J. Cacho, V. Ferreira, Biogenic amine determination in wines using solid-phase extraction: a comparative study, J. Chromatogr. A 1216 (2009) 3398–3401.
[64] N. García-Villar, S. Hernández-Cassou, J. Saurina, Determination of biogenic amines in wines by pre-column derivatization and high-performance liquid chromatography coupled to mass spectrometry, J. Chromatogr. A 1216 (2009) 6387–6393.
[65] A. Dang, J.J. Pesek, M.T. Matyska, The use of aqueous normal phase chromatography as an analytical tool for food analysis: determination of histamine as a model system, Food Chem. 141 (2013) 4226–4230.
[66] I. Basozabal, A. Gomez-Caballero, G. Diaz-Diaz, A. Guerreiro, S. Gilby, M.A. Goicolea, R.J. Barrio, Rational design and chromatographic evaluation of histamine imprinted polymers optimised for solid-phase extraction of wine samples, J. Chromatogr. A 1308 (2013) 45–51.
[67] J. Cai, M. Li, X. Xiong, X. Fang, R. Xu, Detection of histamine in beer by nano extractive electrospray ionization mass spectrometry, J. Mass Spectrom. 49 (2014) 9–12.
[68] M.I. Rodriguez-Naranjo, J.L. Ordóñez, R.M. Callejón, E. Cantos-Villar, M.C. Garcia-Parrilla, Melatonin is formed during winemaking at safe levels of biogenic amines, Food Chem. Toxicol. 57 (2013) 140–146.
[69] C. Ancín-Azpilicueta, A. González-Marco, N. Jiménez-Moreno, Comparative study of the amine concentration in wines obtained from the traditional fermentation and from a more anaerobic fermentation method, LWT - Food Sci. Technol. 43 (2010) 771–776.
[70] C. Berbegal, I. Pardo, S. Ferrer, The use of core-shell high-performance liquid chromatography column technology to improve biogenic amine quantification in wine, J. Sci. Food Agric. 96 (2016) 1556–1561.
[71] F. Patrignani, M. Ndagijimana, N. Belletti, F. Gardini, P. Vernocchi, A.R. Lanciotti, Biogenic amines and ethyl carbamate in primitivo wine: survey of their concentrations in commercial products and relationship with the use of malolactic starter, J. Food Prot. 75 (2012) 591–596.
[72] F. Aflaki, V. Ghoulipour, N. Saemian, S. Sheibani, Biogenic amine contents in non-alcoholic beers: screening and optimization of derivatization, Food Anal. Methods 7 (2014) 713–720.
[73] F. Aflaki, V. Ghoulipour, N. Saemian, M. Salahinejad, A simple method for benzoyl chloride derivatization of biogenic amines for high performance liquid chromatography, Anal. Methods 6 (2014) 1482–1487.
[74] J. Zhong, X. Ye, Z. Fang, G. Xie, N. Liao, J. Shu, et al., Determination of biogenic amines in semi-dry and semi-sweet Chinese rice wines from the Shaoxing region, Food Control 28 (2012) 151–156.
[75] Ö. Özdestan, A. Üren, Biogenic amine content of shalgam (Şalgam): a traditional lactic acid fermented turkish beverage, J. Agric. Food Chem. 58 (2010) 2602–2608.
[76] C. Magwamba, M.I. Matsheka, S. Mpuchane, B.A. Gashe, Detection and quantification of biogenic amines in fermented food products sold in Botswana, J. Food Prot. 73 (2010) 1703–1708.
[77] L. Yañez, J. Saavedra, C. Martínez, A. Córdova, M.A. Ganga, Chemometric analysis for the detection of biogenic amines in chilean cabernet sauvignon wines: a comparative study between organic and nonorganic production, J. Food Sci. 7 (2012) 143–150.
[78] J.Y. Kim, D. Kim, P. Park, H.I. Kang, E.K. Ryu, S.M. Kim, Effects of storage temperature and time on the biogenic amine content and microflora in Korean turbid rice wine, Makgeolli, Food Chem. 128 (2011) 87–92.
[79] A.M. Piasta, A. Jastrzebska, M.P. Krzemiński, T.M. Muzioł, E. Szłyk, New procedure of selected biogenic amines determination in wine samples by HPLC, Anal. Chim. Acta 834 (2014) 58–66.
[80] Y. Cai, Z. Sun, G. Chen, X. Liu, J. You, C. Zhang, Rapid analysis of biogenic amines from rice wine with isotope-coded derivatization followed by high performance liquid chromatography-tandem mass spectrometry, Food Chem. 192 (2016) 388–394.
[81] O. Martínez-Pinilla, Z. Guadalupe, Z. Hernández, B. Ayestarán, Amino acids and biogenic amines in red varietal wines: the role of grape variety, malolactic fermentation and vintage, Eur. Food Res. Technol. 237 (2013) 887–895.
[82] Y.Q. Wang, D.Q. Ye, B.Q. Zhu, G.F. Wu, C.Q. Duan, Rapid HPLC analysis of amino acids and biogenic amines in wines during fermentation and evaluation of matrix effect, Food Chem. 163 (2014) 6–15.
[83] K.J. Huang, C.X. Jin, S.L. Song, C.Y. Wei, Y.M. Liu, J. Li, Development of an ionic liquid-based ultrasonic-assisted liquid-liquid microextraction method for sensitive determination of biogenic amines: application to the analysis of octopamine, tyramine and phenethylamine in beer samples, J. Chromatogr. B 879 (2011) 579–584.

[84] F. Buňka, P. Budinský, M. Čechová, V. Drienovský, V. Pachlová, D. Matoulková, V. Kubáň, L. Buňková, Content of biogenic amines and polyamines in beers from the Czech Republic, J. Inst. Brew. 118 (2012) 213–216.
[85] S. Choi, J.K. Lee, S. Shukla, M. Kim, Physiochemical properties and determination of biogenic amines in korean microbrewery beer products, J. Food Biochem. 36 (2012) 766–773.
[86] M. Mozzon, E. Boselli, M.W. Obiedziński, N.G. Frega, Occurrence of biogenic amines in beers produced with malted organic Emmer wheat (*Triticum dicoccum*), Food Addit. Contam. A 32 (2015) 756–767.
[87] F. Zhang, J. Xue, D. Wang, Y. Wang, H. Zou, B. Zhu, Dynamic changes of the content of biogenic amines in Chinese rice wine during the brewing process, J. Inst. Brew. 119 (2013) 294–302.
[88] K. Henríquez-Aedo, M. Vega, S. Prieto-Rodríguez, M. Aranda, Evaluation of biogenic amines content in chilean reserve varietal wines, Food Chem. Toxicol. 50 (2012) 2742–2750.
[89] A. Pineda, J. Carrasco, C. Peña-Farfal, K. Henríquez-Aedo, M. Aranda, Preliminary evaluation of biogenic amines content in Chilean young varietal wines by HPLC, Food Control 23 (2012) 251–257.
[90] A.C. Manetta, L. Di Giuseppe, R. Tofalo, M. Martuscelli, M. Schirone, M. Giammarco, G. Suzzi, Evaluation of biogenic amines in wine: determination by an improved HPLC-PDA method, Food Control 62 (2016) 351–356.
[91] R.M. Ramos, I.M. Valente, J.A. Rodrigues, Analysis of biogenic amines in wines by salting-out assisted liquid-liquid extraction and high-performance liquid chromatography with fluorimetric detection, Talanta 124 (2014) 146–151.
[92] A. Tassoni, N. Tango, M. Ferri, Comparison of biogenic amine and polyphenol profiles of grape berries and wines obtained following conventional, organic and biodynamic agricultural and oenological practices, Food Chem. 139 (2013).
[93] A. Tassoni, N. Tango, M. Ferri, Polyphenol and biogenic amine profiles of Albana and Lambrusco Grape Berries and Wines obtained following different agricultural and oenological practices, Food Nutr. Sci. 5 (2014) 8–16.
[94] F. Galgano, M. Caruso, G. Perretti, F. Favati, Authentication of Italian red wines on the basis of the polyphenols and biogenic amines, Eur. Food Res. Technol. 232 (2011) 889–897.
[95] H.L. Jiang, L.Y. Ying, S.C. Zhou, M. Ying, W. Shen, D.H. Qiu, Chromatographic determination of biogenic amines in wines after treatment with ionic liquids as novel media, J. Sep. Sci. 34 (2011) 1055–1062.
[96] R. Preti, M.L. Antonelli, R. Bernacchia, G. Vinci, Fast determination of biogenic amines in beverages by a core-shell particle column, Food Chem. 187 (2015) 555–562.
[97] S. Shukla, J.S. Lee, H.K. Park, J.A. Yoo, S.Y. Hong, J.K. Kim, M. Kim, Effect of novel starter culture on reduction of biogenic amines, quality improvement, and sensory properties of *doenjang*, a traditional korean soybean fermented sauce variety, J. Food Sci. 80 (2015).
[98] G. Li, L. Dong, A. Wang, W. Wang, N. Hu, J. You, Simultaneous determination of biogenic amines and estrogens in foodstuff by an improved HPLC method combining with fluorescence labeling, LWT - Food Sci. Technol. 55 (2014) 355–361.
[99] J. Donthuan, S. Yunchalard, S. Srijaranai, Vortex-assisted surfactant-enhanced-emulsification liquid-liquid microextraction of biogenic amines in fermented foods before their simultaneous analysis by high-performance liquid chromatography, J. Sep. Sci. 37 (2014) 3164–3173.
[100] F. Harja, M.D. Stanescu, R. Eder, K. Korntheurer, I. Popescu-Mitroi, D. Haltrich, C. Peterbauer, Determination of biogenic amines from several Romanian wines, Rev. Chim. Buchar. 62 (2011) 304–307.
[101] M.P. Arrieta, M.S. Prats-Moya, Free amino acids and biogenic amines in Alicante Monastrell wines, Food Chem. 135 (2012) 1511–1519.
[102] A. Jeromel, K. Kovačević Ganić, S. Herjavec, M. Mihaljević, A.M. Jagatić Korenika, I. Rendulić, M. Čolić, Concentration of biogenic amines in "pinot noir" wines produced in Croatia, Agric. Conspec. Sci. 77 (2012) 37–40.
[103] G. Garai-Ibabe, A. Irastorza, M.T. Dueñas, P.J. Martín-Álvarez, V.M. Moreno-Arribas, Evolution of amino acids and biogenic amines in natural ciders as a function of the year and the manufacture steps, Int. J. Food Sci. Technol. 48 (2013) 375–381.
[104] A. García-Ruiz, E.M. González-Rompinelli, B. Bartolomé, M.V. Moreno-Arribas, Potential of wine-associated lactic acid bacteria to degrade biogenic amines, Int. J. Food Microbiol. 148 (2011) 115–120.
[105] R. López, C. Tenorio, A.R. Gutiérrez, T. Garde-Cerdán, P. Garijo, L. González-Arenzana, I. López-Alfaro, P. Santamaría, Elaboration of Tempranillo wines at two different pHs. Influence on biogenic amine contents, Food Control 25 (2012) 583–590.
[106] M. García-Marino, Á. Trigueros, T. Escribano-Bailón, Influence of oenological practices on the formation of biogenic amines in quality red wines, J. Food Compost. Anal. 23 (2010) 455–462.
[107] Z. Hu, L. Li, Y. Yuan, T. Yue, Ultrasensitive and simultaneous determination of twenty-one amino acids and amines in culture media, red wine and beer, Food Chem. 158 (2014) 56–65.
[108] I.M. Valente, C.M. Santos, L.M. Gonçalves, J.A. Rodrigues, A.A. Barros, Application of gas-diffusion microextraction for high-performance liquid chromatographic analysis of aliphatic amines in fermented beverages, Anal. Methods 4 (2012) 2569–2573.
[109] S. Jia, Y.P. Kang, J.H. Park, J. Lee, S.W. Kwon, Simultaneous determination of 23 amino acids and 7 biogenic amines in fermented food samples by liquid chromatography/quadrupole time-of-flight mass spectrometry, J. Chromatogr. A 1218 (2011) 9174–9182.
[110] S. Lee, M. Yoo, D. Shin, The identification and quantification of biogenic amines in Korean turbid rice wine, Makgeolli by HPLC with mass spectrometry detection, LWT - Food Sci. Technol. 62 (2015) 350–356.
[111] S. Jia, Y.P. Kang, J.H. Park, J. Lee, S.W. Kwon, Determination of biogenic amines in Bokbunja (*Rubus coreanus* Miq.) wines using a novel ultra-performance liquid chromatography coupled with quadrupole-time of flight mass spectrometry, Food Chem. 132 (2012) 1185–1190.
[112] S. Iljima, Y. Sato, M. Bounoshita, T. Miyaji, D.J. Tognarelli, M. Saito, Optimization of an online post-column derivatization system for UHPLC and its applications to analysis of biogenic amines, Anal. Sci. 29 (2013) 539–545.
[113] J.J. Zhong, N. Liao, T. Ding, X. Ye, D.H. Liu, Liquid chromatographic method for toxic biogenic amines in foods using a chaotropic salt, J. Chromatogr. A 1406 (2015) 331–336.
[114] C. Palermo, M. Muscarella, D. Nardiello, M. Iammarino, D. Centonze, A multiresidual method based on ion-exchange chromatography with conductivity detection for the determination of biogenic amines in food and beverages, Anal. Bioanal. Chem. 405 (2013) 1015–1023.
[115] A. Romano, H. Klebanowski, S. La Guerche, L. Beneduce, G. Spano, M.L. Murat, P. Lucas, Determination of biogenic amines in wine by thin-layer chromatography/densitometry, Food Chem. 135 (2012) 1392–1396.
[116] S.C. Cunha, M.A. Faria, J.O. Fernandes, Gas chromatography-mass spectrometry assessment of amines in port wine and grape juice after fast chloroformate extraction/derivatization, J. Agric. Food Chem. 59 (2011) 8742–8753.
[117] O. Busto, J. Guasch, F. Borrull, Determination of biogenic amines in wine after precolumn derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, J. Chromatogr. A 737 (1996) 205–213.
[118] S. Pandey, Analytical applications of room-temperature ionic liquids: a review of recent efforts, Anal. Chim. Acta 556 (2006) 38–45.
[119] C. Molins-Legua, P. Campins-Falcó, Solid phase extraction of amines, Anal. Chim. Acta 546 (2005) 206–220.
[120] S. Hernández-Cassou, J. Saurina, Derivatization strategies for the determination of biogenic amines in wines by chromatographic and electrophoretic techniques, J. Chromatogr. B 879 (2011) 1270–1281.
[121] A. Önal, A review: current analytical methods for the determination of biogenic amines in foods, Food Chem. 103 (2007) 1475–1486.
[122] R.M. Callejón, A.M. Troncoso, M.L. Morales, Determination of amino acids in grape-derived products: a review, Talanta 81 (2010) 1143–1152.
[123] Ö. Özdestan, A. Üren, A method for benzoyl chloride derivatization of biogenic amines for high performance liquid chromatography, Talanta 78 (2009) 1321–1326.
[124] S. Gómez-Alonso, I. Hermosín-Gutiérrez, E. García-Romero, Simultaneous HPLC analysis of biogenic amines, amino acids, and ammonium ion as aminoenone derivatives in wine and beer samples, J. Agric. Food Chem. 55 (2007) 608–613.
[125] S. Millán, M.C. Sampedro, N. Unceta, M.A. Goicolea, R.J. Barrio, Simple and rapid determination of biogenic amines in wine by liquid chromatography-electrospray ionization ion trap mass spectrometry, Anal. Chim. Acta 584 (2007) 145–152.
[126] Y.Y. Guo, Y.P. Yang, Q. Peng, Y. Han, Biogenic amines in wine: a review, Int. J. Food Sci. Tech. 50 (2015) 1523–1532.
[127] P. Ginterová, J. Marák, A. Stanová, V. Maier, J. Sevcík, D. Kaniansky, Determination of selected biogenic amines in red wines by automated on-line combination of capillary isotachophoresis-capillary zone electrophoresis, J. Chromatogr. B 904 (2012) 135–139.
[128] D. An, Z. Chen, J. Zheng, S. Chen, L. Wang, Z. Huang, L. Weng, Determination of biogenic amines in oysters by capillary electrophoresis coupled with electrochemiluminescence, Food Chem. 168 (2015) 1–6.
[129] J. Xie, Y. Li, J. Zhang, L. Zeng, D. Lu, Y. Liu, Y. Yang, C. Sun, Simultaneous determination of four aliphatic amines in aquatic products by ultrasound-assisted dispersive liquid-liquid microextraction coupled with high performance capillary electrophoresis, Anal. Methods 6 (2014) 5140–5146.
[130] D. Daniel, V.B. Dos Santos, D.T.R. Vidal, C.L. do Lago, Determination of biogenic amines in beer and wine by capillary electrophoresis-tandem mass spectrometry, J. Chromatogr. A 1416 (2015) 121–128.
[131] N. Dossi, R. Toniolo, A. Pizzariello, S. Susmel, G. Bontempelli, A modified electrode for the electrochemical detection of biogenic amines and their amino acid precursors separated by microchip capillary electrophoresis, Electrophoresis 32 (2011) 906–912.
[132] A. Jastrzębska, A. Piasta, E. Szłyk, Simultaneous determination of selected biogenic amines in alcoholic beverage samples by isotachophoretic and chromatographic methods, Food Addit. Contam. A 31 (2014) 83–92.
[133] S. Uzaşçi, S. Başkan, F. Bedia Erim, Biogenic amines in wines and pomegranate molasses-a non-ionic micellar electrokinetic chromatography Assay with laser-induced fluorescence detection, Food Anal. Methods 5 (2012) 104–108.
[134] I. Basozabal, A. Guerreiro, A. Gomez-Caballero, M. Aranzazu Goicolea, R.J. Barrio, Direct potentiometric quantification of histamine using solid-phase imprinted nanoparticles as recognition elements, Biosens. Bioelectron. 58 (2014) 138–144.
[135] B. Bóka, N. Adányi, J. Szamos, D. Virág, A. Kiss, Putrescine biosensor based on putrescine oxidase from *Kocuria rosea*, Enzyme Microb. Tech. 51 (2012) 258–262.
[136] B. Batra, S. Lata, R. Devi, S. Yadav, C.S. Pundir, Fabrication of an amperometric tyramine biosensor based on immobilization of tyramine oxidase on AgNPs/

L-Cys-modified Au electrode, J. Solid State Electr. 16 (2012) 3869–3876.
[137] S. Lata, S. Yadav, R. Bhardwaj, C.S. Pundir, Amperometric determination of tyramine in sauce and beer by epoxy resin biocomposite membrane bound tyramine oxidase, Sens. Instrum. Food Qual 5 (2011) 104–110.
[138] T. Ramon-Marquez, A.L. Medina-Castillo, A. Fernandez-Gutierrez, J.F. Fernandez-Sanchez, Novel optical sensing film based on a functional nonwoven nanofibre mat for an easy, fast and highly selective and sensitive detection of tryptamine in beer, Biosens. Bioelectron. 79 (2016) 600–607.
[139] W. Henao-Escobar, L. Del Torno-de Román, O. Domínguez-Renedo, M.A. Alonso-Lomillo, M.J. Arcos-Martínez, Dual enzymatic biosensor for simultaneous amperometric determination of histamine and putrescine, Food Chem. 190 (2016) 818–823.
[140] M. Di Fusco, R. Federico, A. Boffi, A. Macone, G. Favero, F. Mazzei, Characterization and application of a diamine oxidase from *Lathyrus sativus* as component of an electrochemical biosensor for the determination of biogenic amines in wine and beer, Anal. Bioanal. Chem. 401 (2011) 707–716.
[141] C.I.L. Justino, A.C. Freitas, R. Pereira, A.C. Duarte, T.A.P. Rocha Santos, Recent developments in recognition elements for chemical sensors and biosensors, TrAC- Trend. Anal. Chem. 68 (2015) 2–17.
[142] R.C. Alves, M.F. Barroso, M.B. González-García, M.B.P.P. Oliveira, M.C. Delerue-Matos, New trends in food allergens detection: towards biosensing strategies, Crit. Rev. Food Sci. (2015), http://dx.doi.org/10.1080/10408398.2013.831026.
[143] A. Marcobal, M.C. Polo, P.J. Martín-Álvarez, M.V. Moreno-Arribas, Biogenic amine content of red Spanish wines: comparison of a direct ELISA and an HPLC method for the determination of histamine in wines, Food Res. Int. 38 (2005) 387–394.
[144] L. Luo, Z.L. Xu, J.Y. Yang, Z.L. Xiao, Y.J. Li, R.C. Beier, Y.M. Sun, H.T. Lei, H. Wang, Y.D. Sheng, Synthesis of novel haptens and development of an enzyme-linked immunosorbent Assay for quantification of histamine in foods, J. Agric. Food Chem. 62 (2014) 12299–12308.
[145] S. Hernández-cassou, J. Saurina, Determination of histamine in wine samples by flow-injection analysis and multivariate calibration by flow-injection analysis and multivariate calibration, Anal. Lett. 46 (2013) 1758–1768.
[146] P. Herbert, M.J. Cabrita, N. Ratola, O. Laureano, A. Alves, Free amino acids and biogenic amines in wines and musts from the Alentejo region. Evolution of amines during alcoholic fermentation and relationship with variety, subregion and vintage, J. Food Eng. 66 (2005) 315–322.
[147] S. Loret, P. Deloyer, G. Dandrifosse, Levels of biogenic amines as a measure of the quality of the beer fermentation process: data from Belgian samples, Food Chem. 89 (2005) 519–525.
[148] P. Kalač, J. Šavel, M. Křížek, T. Pelikánová, M. Prokopová, Biogenic amine formation in bottled beer, Food Chem. 79 (2002) 431–434.
[149] J.M. Landete, S. Ferrer, I. Pardo, Biogenic amine production by lactic acid bacteria, acetic bacteria and yeast isolated from wine, Food Control 18 (2007) 1569–1574.
[150] T. Frank, B. Scholz, S. Peter, K.H. Engel, Metabolite profiling of barley: influence of the malting process, Food Chem. 124 (2011) 948–957.
[151] M. Arbulu, M.C. Sampedro, A. Gómez-Caballero, M.A. Goicolea, R.J. Barrio, Untargeted metabolomic analysis using liquid chromatography quadrupole time-of-flight mass spectrometry for non-volatile profiling of wines, Anal. Chim. Acta 858 (2015) 32–41.
[152] S. Pérez-Magariño, M. Ortega-Heras, L. Martínez-Lapuente, Z. Guadalupe, B. Ayestarán, Multivariate analysis for the differentiation of sparkling wines elaborated from autochthonous Spanish grape varieties: volatile compounds, amino acids and biogenic amines, Eur. Food Res. Technol. 236 (2013) 827–841.
[153] H. Trufelli, P. Palma, G. Famiglini, A. Cappiello, An overview of matrix effects in liquid chromatography-mass spectrometry, Mass Spectrom. Rev. 30 (2011) 491–509.

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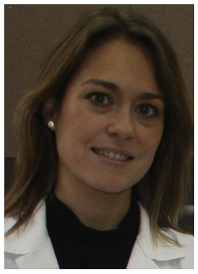

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CAPÍTULO II

Resumen

Este capítulo se centra en la adaptación y validación de un método cromatográfico para determinar aminas biógenas, aminoácidos y amonio en puré de fresa y productos obtenidos tras su fermentación glucónica. Las muestras fueron derivatizadas con etoximetilenmalonato de dietilo (DEEMM) como reactivo derivatizante precolumna y se analizaron con un cromatógrafo líquido acoplado a un detector de matriz de diodo.

El método analítico fue adaptado y validado exitosamente para la determinación de 8 aminas biógenas (histamina, tiramina, putrescina, cadaverina, agmatina, espermidina, triptamina y feniletilamina), 22 aminoácidos y el ión amonio en puré de fresa y sus fermentados de ácido glucónico en términos de linealidad, sensibilidad (LD y LQ) y precisión (repetibilidad y precisión intermedia) de acuerdo con los criterios establecidos por AOAC (AOAC, 1993).

El método fue empleado en primer lugar para determinar y comparar el perfil de los compuestos nitrogenados en diferentes procesos de fermentación glucónica llevados a cabo mediante cultivo superficial por distintas bacterias acéticas: *Acetobacter malorum,Gluconobacter oxydans y Gluconobacter japonicus.* Respecto a las aminas biógenas, se puede afirmar que estos fermentados glucónicos de fresa pueden ser útiles para la elaboración de productos alimenticios ya que sus concentraciones estaban por debajo de los límites de detección, y por tanto, por debajo de los niveles que causan efectos perjudiciales en los consumidores. Por otro lado, el perfil de aminoácidos, específicamente, glutamina, alanina, arginina, prolina, ácido glutámico, asparagina-serina, isoleucina, leucina y fenilalanina, ha mostrado ser un parámetro que permite discriminar los fermentados de acuerdo a la cepa empleada (*A. malorum*, *G. oxydans* y *G. japonicus*). De estas tres cepas, *G. japonicus* fue la que produjo mayores cambios en la concentración de aminoácidos respecto las otras. Además, fue la cepa que más glucosa consumía, manteniendo los niveles de fructosa procedentes del sustrato.

Así pues, *G. japonicus* fue la cepa seleccionada para la elaboración de la bebida ya que mostró una mayor actividad durante la fermentación. De esta forma, se evaluó posteriormente durante las fermentaciones con cultivo sumergido en fermentadores a escala piloto, Este tipo de fermentación proporciona una alternativa mucho más rápida que la fermentación con cultivo superficial y más interesante para aplicarla a una escala industrial.

Las fermentaciones se monitorizaron en términos de pH, acidez total, concentraciones de glucosa, fructosa y ácido glucónico así como concentraciones de amina biógenas, aminoácidos y

amonio. Al igual que en la fermentación con cultivo superficial, no se detectaron aminas biógenas en las muestra finales, por lo que se garantiza la seguridad de este producto para el consumo humano. La mayoría de los aminoácidos no presentaron cambios significativos, excepto lisina e histidina, las cuales aumentaron en el producto final. Sin embargo, aunque los fermentados presentaban pocas diferencias en el perfil de aminoácidos e ion amonio, el análisis de componentes principales (PCA) fue capaz separar dos grupos, uno para las muestras iniciales y otro con las muestras finales usando como variables lisina, fenilalanina, ácido glutámico, isoleucina, histidina, ácido aspártico, leucina, alanina e ion amonio.

Artículo 2

Impact of gluconic fermentation of strawberry on amino acids and biogenic amines profile.

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Food Chemistry
178 (2015) 221–228

Q1; 6/130 (JCR-ISI, Food Science & Technology, 2016)
Impact Factor: 4.529

Food Chemistry 178 (2015) 221–228

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Impact of gluconic fermentation of strawberry using acetic acid bacteria on amino acids and biogenic amines profile

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ARTICLE INFO

Article history:
Received 18 September 2014
Received in revised form 12 December 2014
Accepted 19 January 2015
Available online 28 January 2015

Keywords:
HPLC
DEEMM
Nitrogen compounds
Beverages
Strawberry products
Acetic acid bacteria

ABSTRACT

This paper studies the amino acid profile of beverages obtained through the fermentation of strawberry purée by a surface culture using three strains belonging to different acetic acid bacteria species (one of *Gluconobacter japonicus*, one of *Gluconobacter oxydans* and one of *Acetobacter malorum*). An HPLC–UV method involving diethyl ethoxymethylenemalonate (DEEMM) was adapted and validated. From the entire set of 21 amino acids, multiple linear regressions showed that glutamine, alanine, arginine, tryptophan, GABA and proline were significantly related to the fermentation process. Furthermore, linear discriminant analysis classified 100% of the samples correctly in accordance with the microorganism involved. *G. japonicus* consumed glucose most quickly and achieved the greatest decrease in amino acid concentration. None of the 8 biogenic amines were detected in the final products, which could serve as a safety guarantee for these strawberry gluconic fermentation beverages, in this regard.

1. Introduction

Strawberry (*Fragaria x ananassa* Duch.) is one of the most economically important fresh and processed fruits (Hancock, Sjulin, & Lobos, 2008) and a source of bioactives (Álvarez-Fernández, Hornedo-Ortega, Cerezo, Troncoso, & García-Parrilla, 2014a; Cerezo, Cuevas, Winterhalter, Garcia-Parrilla, & Troncoso, 2010; Stürtz, Cerezo, Cantos-Villar, & Garcia-Parrilla, 2011). Hence, there is a wide variety of processed strawberry products, such as purée, jams, juices, beverages, fruit preparations, etc. (Fügel, Carle, & Schieber, 2005; Hui et al., 2006). Recently, strawberry fermented products, such as wines and vinegars, have been produced as a good solution for using strawberry surpluses and as an alternative method for conserving this perishable fruit (Hidalgo, Torija, Mas, & Mateo, 2013; Ubeda et al., 2013).

Gluconic acid is abundantly available in grains, fruits and other foodstuffs, such as rice, meat, dairy products, honey and fermented products like wine and vinegar. It is a mild organic acid, which has applications in the food industry (Deppenmeier, Hoffmeister, & Prust, 2002; Ramachandran, Fontanille, Pandey, & Larroche, 2006; Singh & Kumar 2007). It is produced from glucose by different microorganisms, which include bacteria, yeast and some ectomycorrhizal fungus. Among them, some genera of the family *Acetobacteraceae*, such as *Gluconobacter*, are used industrially to produce gluconic acid (Deppenmeier & Ehrenreich, 2009; Ramachandran et al., 2006). There are several works that have studied gluconic acid fermentations. However, most of them are focused on biotechnology and its applications (Deppenmeier et al., 2002; Gupta, Singh, Qazi, & Kumar, 2001; Ramachandran, Fontanille, Pandey, & Larroche, 2006; Singh & Kumar, 2007) and reports focusing on the gluconic fermentation of fruits are scarce.

Acetic acid bacteria (AAB) can utilize a wide range of compounds as sources of nitrogen, from simple inorganic compounds to complex compounds, including amino acids (Merrick & Edwards, 1995). *Gluconobacter* strains are able to grow using ammonium ion as their sole source of nitrogen (Deppenmeier & Ehrenreich, 2009; Gupta et al., 2001). However, AAB have been shown to consume amino acids during the conversion of ethanol into acetic acid (Callejón, Troncoso, & Morales, 2010). Hence, free amino acids present in the medium could also be a good source of nitrogen for these bacteria, in addition to ammonium ions. The amino acid content of fruits and fruit derived products is studied since they contribute to the final aroma and taste, among other properties (Mandrioli, Mercolini, & Raggi, 2013).

Furthermore, some biogenic amines can be directly formed from amino acids by decarboxylation. These compounds can be formed and degraded during the normal metabolism of living organisms, although they have been quantified especially in fermented foods and beverages such as cheeses, dry fermented sausages or wine (Ancín-Azpilicueta, González-Marco, & Jiménez-Moreno, 2008; ten Brink, Damink, Joosten, & Huis In't Veld, 1990;

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http://dx.doi.org/10.1016/j.foodchem.2015.01.085
0308-8146/

Kirschbaum, Rebscher, & Brückner, 1999). High concentrations of biogenic amines in the final products could be due to poor quality raw materials, contamination, or to food processing and storage under unsuitable conditions (Önal, 2007; ten Brink, Damink, Joosten, & Huis In't Veld, 1990). Biogenic amines, in particular histamine and tyramine, can cause health problems when are present in foods in a high concentration (ten Brink, Damink, Joosten, & Huis In't Veld, 1990). These compounds could cause wide effects on consumer such as headache, inflammations, irritation, hypertension, and hypotension (Ancín-Azpilicueta et al., 2008; ten Brink, Damink, Joosten, & Huis In't Veld, 1990). The European legislation does not have a biogenic amines threshold, but the European Food Safety Authority (EFSA) has elaborated a scientific opinion on the risk associated with their formation in fermented products (European Food Safety Authority (EFSA) Panel on Biological Hazards (BIOHAZ), 2011).

Several techniques have been developed for analyzing amino acids and biogenic amines in foods (Callejón et al. 2010; Hernández-Orte, Ibarz, Cacho, & Ferreira, 2003; Önal, 2007; Peña-Gallego, Hernández-Orte, Cacho, & Ferreira, 2012). Nevertheless, the analytical technique most frequently employed for the determination of amino acids and biogenic amines is HPLC with C18 reverse-phase columns (Peña-Gallego et al., 2012). This method is less time-consuming than other techniques and the instrumentation used is simple (Hernández-Orte et al., 2003). The direct detection of amino acids by HPLC yields matrix interferences (Callejón et al., 2008) and biogenic amines do not have good absorption properties in the visible, ultraviolet or fluorescence wavelength ranges (Peña-Gallego, Hernández-Orte, Cacho, & Ferreira, 2009). For these reasons, the determination of these compounds requires a chemical derivatization to improve detection limits and to avoid matrix interference (Callejón et al., 2010; Gómez-Alonso, Hermosín-Gutiérrez, & García-Romero, 2007; Peña-Gallego et al., 2012). The reagents most widely used are 2,2-dihydroxy-1,3-indanedione (Ninhydrin), dansyl chloride (DnsCl), dabsyl Chloride (DbsCl), phenylisothiocyanate (PITC), o-phthaldialdehyde (OPA), 6-aminoquinolyl N-hydroxysuccinimidyl carbamate (AQC) and diethyl ethoxymethylenemalonate (DEEMM), among others (Callejón et al., 2010; Peña-Gallego et al., 2012). Some of these techniques have been able to determine amino acids and biogenic amines simultaneously, such as the method proposed by Gómez-Alonso et al. (2007), which used DEEMM as the derivatization agent to increase the specific absorbance of the analytes, followed by reverse phase HPLC and UV–vis photodiode array detection. This method has been proposed for wines and beers.

The aims of this study were: (a) to adapt an analytical method to determine the profile of amino acids, biogenic amines and ammonium ion in different gluconic acid fermented products and in the starting substrate (strawberry purée) by HPLC using DEEMM as the derivatization agent; (b) to study the differences in amino acid consumption by the different AAB strains employed; (c) to verify whether the fermented products can be discriminated or grouped according to the strain that performed the fermentation, taking the amino acid profiles as variables, and (d) to check whether these fermented beverages are safe for human consumption by determining the concentrations of biogenic amines.

2. Materials and methods

2.1. Reagents and standards

Most of the amino acid standards were purchased from Fluka (Buchs, Switzerland). The aspartic acid, glutamic acid, histidine, alanine, lysine, γ-aminobutiric acid (GABA), biogenic amines, ammonium sulphate, diethyl ethoxymethylenemalonate, acetic acid glacial, boric acid, 2-aminoadipic acid (internal standard) and sodium azide were supplied by Sigma–Aldrich (Steinheim, Germany). The glycine, ornithine, methanol (HPLC grade) and acetonitrile (HPLC grade) were acquired from Merck (Darmstadt, Germany). The sodium acetate and sodium hydroxide were obtained from Panreac (Castellar del Vallès, Barcelona). The ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

The stock standard solutions were prepared individually by dissolving the pure compounds in HCl 0.1 N. The calibration solutions were prepared by diluting the stock standard solutions with water.

2.2. Samples

We analyzed samples of the fermentation of strawberry purée using a surface cultures of acetic acid bacteria (AAB), supplied by HUDISA, S.A. (Lepe, Spain). These gluconic fermentations were conducted in the laboratories of the Biochemistry and Biotechnology Department (Facultat d'Enologia, Universitat Rovira i Virgili. Tarragona, Spain). These fermentations were carried out with different AAB strains: one of *Acetobacter malorum* (3 samples), one of *Gluconobacter oxydans* (3 samples) and one of *Gluconobacter japonicus* (2 samples). The initial substrate used for these processes was also studied. AAB were grown in a GY medium (1% yeast extract and 5% glucose) and incubated at 28 °C with stirring. The fermentation substrate consisted of mixing 90% strawberry purée with 10% rectified concentrated must (Concentrados Pallejà, Riudoms, Spain). For each fermentation, 500 mL of the substrate were inoculated with 2×10^6 cell/mL of the AAB strains in a 1 L Erlenmeyer flask. Fermentations were performed at 28 °C with a stirring speed of 128 rpm. In all cases the fermentations were considered finished after 10 days of fermentation, when more than 90% of the initial glucose had been consumed. Only *G. japonicus* strain practically exhausted glucose after the 10 days. All samples were frozen immediately after sampling. Table 1 displays the sample codes and their concentrations in glucose, fructose and gluconic acid.

2.3. Sample preparation

First, 2 mL of sample were centrifuged at 6000 rpm for 15 min (Eppendorf centrifuge 5415R, Hamburg, Germany). The

Table 1
Samples codes and glucose, fructose and gluconic acid concentration in strawberry purée and gluconic fermented.

Samples		Codex	Glucose (g/L)	Fructose (g/L)	Gluconic acid (g/L)
Strawberry purée		*SP*	62	62	–
Strawberry gluconic acid fermentation beverages (SGFB)	*Acetobacter malorum* (SGFAM)	*SGFAM1*	4.74	51.72	49.91
		SGFAM2	6.68	54.69	45.73
		SGFAM3	2.25	51.33	47.78
	Gluconobacter oxydans (SGFGO)	*SGFGO1*	4.74	56.96	51.95
		SGFGO2	4.12	50.47	46.16
		SGFGO3	2.57	54.22	49.48
	Gluconobacter japonicus (SGFGJ)	*SGFGJ1*	0.93	50.94	52.38
		SGFGJ2	0.78	47.49	47.95

derivatization of amino acids and biogenic amines was performed by diethyl ethoxymethylenemalonate (DEEMM). For this, 700 μL of borate buffer 1 M (pH = 9), 300 μL of methanol, 400 μL of standard or sample, 10 μL of internal standard (L-2-aminoadipic acid, 1 g/L) and 12 μL of DEEMM were mixed in a covered vial, which was introduced into an ultrasound bath for 30 min. Later, the sample was heated at 70 °C for 2 h to allow the complete degradation of excess DEEMM and reagent byproducts (Gómez-Alonso et al. 2007). All samples were filtered through a membrane filter with a mean pore size of 0.45 μm (Millipore) prior to use.

2.4. Equipment

HPLC analysis was carried out in a Waters HPLC system consisting of a Waters 717 autosampler injector and a Waters 1525 Binary HPLC pump system controller connected to a Waters 996 photodiode array detector. Data treatment was performed in a Waters Millennium data station. The column consisted of a LiChroCART® 250-4 LiChrospher® 100 RP-18 (5 μm, 250 × 4.6 mm) from Merck (Darmstadt, Germany) and a 4.0 × 3.0 mm guard column from Analytical Phenomenex (Torrance, CA, USA). The column was thermostatized at 45 °C in a column header module controlled by Waters TCM HPLC Temperature Controller. The gradient program employed is shown in Table 2; it was similar to the one used by Gómez-Alonso et al. (2007). The injection volume was 10 μL and the separation was obtained at a flow rate of 0.9 mL/min. Mobile phase A consisted of a 25 mM acetate buffer (pH 5.8) with 0.02% sodium azide, and mobile phase B was an 80:20 mixture of acetonitrile and methanol. A photodiode array detector monitored at 280 and 269 nm was used for detection. All mobile phases were filtered through a membrane filter with a mean pore size of 0.45 μm (Millipore) prior to use.

Table 2
Eluent gradient for HPLC method.

Time (min)	A (%)	B (%)
0	90	10
20	90	10
26	87	13
32	83	17
34	83	17
43	75	25
48	75	25
53	70	30
58	70	30
65	60	40
72	28	72
75	25	75
77	20	80
79	20	80
85	0	100

2.5. Statistical analysis

All statistical analyses were performed using the Statistica software (StatSoft Inc., 2004). One-way ANOVA was performed to evaluate significant differences between types of samples (significance levels $p < 0.05$). Multiple linear regression (MLR) was performed to evaluate relationships between amino acid concentrations and glucose consumption.

In addition, Principal Component Analysis (PCA) followed by Lineal Discriminant Analysis (LDA) was employed to evaluate whether the profiles of amino acids and biogenic amines were different enough to distinguish between the gluconic acid fermentations analyzed.

Table 3
Results of regression analysis of calibration curves, LOD, LOQ, repeatability and intermediate precision.

Compounds	λ (nm)	RT (min)	Linear range (mg/L)	Equation $y = ax + b$	R^2	LOD (mg/L)	LOQ (mg/L)	Repeatability ($n = 5$)		Intermediate precision ($n = 5$)	
								Mean (mg/L)	RSD (%)	Mean (mg/L)	RSD (%)
Aspartic acid	280	3.8	0.5–100	$y = 0.055x + 0.1715$	0.9929	0.15	0.45	41.94	1.27	41.84	2.47
Glutamic acid	280	4.4	0.5–150	$y = 0.0542x + 0.1309$	0.997	0.10	0.30	35.98	0.73	35.79	2.58
Asparagine	280	7.4	0.5–800	$y = 0.0382x + 0.1171$	0.9964	0.15	0.45	62.34	2.14	62.14	1.18
Serine	280	7.4	0.5–100	$y = 0.073x + 0.0107$	0.9997	0.15	0.45	55.78	0.49	55.83	0.11
Glutamine	280	8.3	5–800	$y = 0.0484x + 0.5199$	0.9985	0.10	0.30	26.70	1.74	25.43	2.65
Histidine	280	10.4	0.5–100	$y = 0.0529x - 0.0306$	0.9977	0.15	0.45	43.14	0.29	43.37	0.44
Glycine	280	11.9	0.5–100	$y = 0.1127x + 0.0299$	0.9999	0.05	0.15	36.84	1.02	36.55	2.30
Threonine	280	12.2	1–100	$y = 0.0611x - 0.0267$	0.9987	0.05	0.15	35.68	2.06	35.36	1.51
Alanine	280	19.8	1–150	$y = 0.0689x + 0.0793$	0.9987	0.05	0.15	53.60	1.95	52.66	1.32
Arginine	280	21.2	5–100	$y = 0.0383x + 0.0045$	0.9962	0.05	0.15	39.86	1.57	40.03	3.98
GABA	280	22	0.5–150	$y = 0.0678x - 0.0427$	0.9996	0.05	0.15	45.29	1.01	44.03	4.68
Proline	280	34.8	5–3000	$y = 0.025x - 0.3434$	0.9973	0.3	0.90	66.97	1.27	58.01	13.90
Tyrosine	280	35.6	1–100	$y = 0.021x + 0.7793$	0.99	0.05	0.15	61.31	2.81	66.06	2.17
Valine	280	43.4	1–100	$y = 0.0624x + 0.169$	0.9988	0.05	0.15	41.45	1.51	40.73	1.25
Methionine	280	44	0.5–100	$y = 0.0339x - 0.0147$	0.9975	0.05	0.15	60.75	0.60	60.17	0.47
Cysteine	280	46.7	0.5–100	$y = 0.0267x + 0.0521$	0.997	0.05	0.15	43.82	6.06	48.49	7.92
Isoleucine	280	49.7	0.5–100	$y = 0.0467x + 0.0901$	0.9957	0.05	0.15	15.94	7.89	14.51	3.21
Tryptophan	280	50.3	0.5–100	$y = 0.0818x - 0.0572$	0.9974	0.05	0.15	45.76	1.14	46.14	4.07
Leucine	280	50.9	0.5–100	$y = 0.0633x + 0.2051$	0.9993	0.05	0.15	44.18	2.27	46.53	9.34
Phenylalanine	280	51.5	1–100	$y = 0.0383x + 0.0011$	0.9994	0.05	0.15	97.25	1.54	95.31	2.72
Ornithine	280	56.9	0.5–100	$y = 0.0256x + 0.037$	0.9976	0.05	0.15	52.79	1.55	51.00	3.32
Lysine	280	60.3	0.5–100	$y = 0.077x + 0.0198$	0.9997	0.05	0.15	40.04	0.76	39.92	0.75
Histamine	280	35.3	0.5–100	$y = 0.0286x + 0.0246$	0.9936	0.05	0.15	87.14	4.89	78.50	4.84
Agmatine	280	38.7	0.5–100	$y = 0.025x + 0.1354$	0.9921	0.05	0.15	23.08	7.62	22.63	4.47
Spermidine	280	60.7	0.5–100	$y = 1.0326x - 0.0169$	0.9991	0.10	0.30	1.97	0.33	1.96	0.98
Tyramine	280	71.5	0.5–100	$y = 0.0627x - 0.0644$	0.995	0.05	0.15	42.83	1.44	41.60	2.45
Putrescine	280	74.9	0.5–100	$y = 0.0582x + 0.151$	0.9958	0.05	0.15	52.82	1.61	51.94	1.35
Tryptamine	280	75.1	0.5–100	$y = 0.055x + 0.1047$	0.9975	0.05	0.15	25.49	1.04	25.15	2.55
Cadaverine	280	75.9	0.5–100	$y = 0.0561x + 0.1219$	0.9943	0.05	0.15	43.75	1.45	42.98	1.15
Phenylethylamine	280	75.9	0.5–100	$y = 0.0461x + 0.0044$	0.9998	0.05	0.15	44.77	0.54	43.36	3.19
Ammonium	269	37.8	1–100	$y = 0.0714x + 0.0631$	0.9996	0.05	0.15	20.25	3.84	20.41	5.27

RT: Retention time, R^2: Correlation coefficients, LOD: Limits of detection, RSD: Relative standard deviation.

3. Results and discussion

3.1. Method validation

The analytical method used to perform this work was originally developed by Gómez-Alonso et al. (2007) to determine amino acids in wines and beers. Slight modifications in gradient and temperature were performed to obtain better peak resolution in the strawberries and their fermented products. Thus, the modified method was validated in terms of linearity, sensitivity (detection and quantification limits) and precision (repeatability and intermediate precision) response according to AOAC criteria (AOAC, 1993). Table 3 displays these validation parameters. The linear range was obtained using a value close to the limit of quantification (LOQ) as the lowest concentration. Thus, the lowest point of the linear range was lower than that described by Gómez-Alonso et al. (2007) in most cases. In all the analytes, R^2 were above 0.99, showing a linear relationship between standard concentration and detector response.

Room temperature in our laboratory oscillates widely. Therefore, we set the temperature at 45 °C to achieve repeatability and intermediate precision within the AOAC limits (AOAC, 1993).

Due to the high concentration of asparagine and its proximity to serine, peak overlapping could not be prevented, so the two were quantified together (asparagine–serine) as previously described (Hermosín, Chicón, & Dolores Cabezudo, 2003).

3.2. Sample analysis

The proposed method was applied to determine amino acids and biogenic amines in strawberry purées and beverages obtained after gluconic fermentation.

Table 4 displays amino acid concentration in samples obtained by surface culture fermentation with different AAB strains. A total of 31 compounds were determined, including 22 amino acids, 8 biogenic amines and ammonium ion.

Asparagine-serine, alanine and glutamine were the major amino acids in the substrate as shown Fig. 1, which is in accordance to the strawberry amino acids profile (Moing et al., 2001; Perez, Rios, Sanz, & Olias, 1992). On the other hand, the amino acid profiles changed after gluconic fermentation (Fig. 2). In general, most amino acids followed a similar trend, and their concentration changed during fermentations with the different AAB strains. In order to search for a relationship between amino acids and glucose consumption, we performed a correlation matrix analysis, which revealed the most significant correlations. Hence, we selected glutamine, alanine, arginine, GABA, proline and tryptophan for subsequent multiple linear regression analysis. We tested 3 methods: standard, forward stepwise and backward stepwise inclusion of variables, and obtained an R^2 of 0.9874 for standard and forward stepwise analysis and an R^2 of 0.9807 for backward stepwise analysis. The following variables were included in the forward stepwise analysis (in order): alanine, arginine, glutamine, tryptophan and

Table 4
Concentration of amino acids, biogenic amines and ammonium ion in strawberry purée and gluconic fermented products.

Compounds	Mean concentrations (mg/L) ± RSD			
	SP	SGFAM	SGFGO	SGFGJ
Aspartic acid	45.98[b,c] ± 0.79	34.10[a] ± 1.53	31.20[a,d] ± 1.29	38.77[c] ± 4.14
Glutamic acid	58.53[d] ± 0.20	58.39[d] ± 1.98	56.38[d] ± 2.45	48.43[a,b,c] ± 3.50
Asparagine-serine	717.17[d] ± 12.09	711.73 ± 21.89	654.92 ± 35.04	594.00[a] ± 18.56
Glutamine	102.96[b,c,d] ± 0.88	30.49[a,d] ± 0.64	25.11[a,d] ± 1.37	18.35[a,b,c] ± 2.18
Histidine	22.34[b,d] ± 0.42	17.74[a,d] ± 1.89	18.34[d] ± 2.31	15.21[a,b,c] ± 0.87
Glycine	1.48[b] ± 0.32	2.28[a] ± 0.36	2.00 ± 1.17	1.82 ± 1.21
Threonine	37.86 ± 2.73	36.52 ± 1.58	33.75 ± 2.22	34.24 ± 1.57
Alanine	113.93[b,c,d] ± 3.13	28.26[a,d] ± 0.46	17.56[a] ± 2.15	19.97[a,b] ± 2.52
Arginine	6.43[b,c,d] ± 0.91	59.35[a] ± 1.69	63.16[a] ± 3.01	63.73[a] ± 0.72
GABA	1.89[b,c,d] ± 0.89	5.12[a,c] ± 0.74	6.73[a,b] ± 1.31	5.99[a] ± 0.87
Proline	13.74[b,c,d] ± 1.34	36.55[a] ± 2.78	41.46[a] ± 2.17	38.96[a] ± 2.35
Tyrosine	1.58[c,d] ± 1.20	1.18 ± 0.51	ND	ND
Valine	6.16[d] ± 0.82	4.10[d] ± 0.58	3.50[d] ± 0.74	1.99[a,b,c] ± 0.19
Methionine	10.12 ± 1.11	11.47 ± 1.37	12.47 ± 2.22	12.44 ± 1.44
Cysteine	ND	ND	ND	ND
Isoleucine	4.69[d] ± 0.53	3.34[d] ± 1.12	4.25[d] ± 1.20	0.34[a,b,c] ± 1.13
Tryptophan	2.11[b,c,d] ± 0.09	0.70[a] ± 0.12	0.90[a] ± 0.44	0.70[a] ± 0.15
Leucine	0.64 ± 0.55	0.83 ± 1.47	0.62 ± 1.04	0.65 ± 0.80
Phenylalanine	7.97[d] ± 0.78	9.65 ± 1.95	9.18 ± 1.82	9.87[a] ± 0.70
Ornithine	4.08[d] ± 0.13	2.09 ± 1.50	1.48 ± 0.50	ND
Lysine	1.6 ± 0.36	1.83 ± 0.59	2.59 ± 1.06	1.69 ± 0.71
Histamine	ND	ND	ND	ND
Agmatine	ND	ND	ND	ND
Spermidine	ND	ND	ND	ND
Tyramine	ND	ND	ND	ND
Putrescine	ND	ND	ND	ND
Tryptamine	ND	ND	ND	ND
Cadaverine	ND	ND	ND	ND
Phenylethylamine	ND	ND	ND	ND
Ammonium	39.51[c,d] ± 0.17	49.04[d] ± 5.19	57.58[a,d] ± 3.63	64.64[a,b,c] ± 2.26

ND: non detected.
RSD: Relative standard deviation.
Codes of samples are explained in Table 1.
[a] Shows significant differences between substrate and other samples according to ANOVA test ($p < 0.05$).
[b] Shows significant differences between *A. malorum* and other samples according to ANOVA test ($p < 0.05$).
[c] Shows significant differences between *G. oxydans* and other samples according to ANOVA test ($p < 0.05$).
[d] Shows significant differences between *G. japonicus* and other samples according to ANOVA test ($p < 0.05$).

GABA. However, only glutamine and arginine were included in the backward stepwise analysis model.

Glutamine, alanine and tryptophan showed a significant decrease. These amino acids were mostly consumed by AAB. They have already been shown to be a good nitrogen source for microorganisms such as AAB and yeast (Arias-Gil, Garde-Cerdán, & Ancín-Azpilicueta, 2007; Joubert, Bayens, & De Ley, 1961; Sánchez & Demain, 2002).

Conversely, other amino acids, such as arginine, GABA and proline, increased significantly during fermentation. Among them, the increase of arginine was the most pronounced. A probable cause of the arginine increase in the final beverages is that this amino acid is known to be stored in microorganism vacuole under nitrogen-available conditions, and it is left in the media after autolysis (Carrasco & Pérez-Ortín, 2003; Leigh & Dodsworth, 2007). Increases in GABA have been previously reported following the fermentation of blackberry with *Lactobacillus brevis* (Kim, Lee, Ji, Lee, & Hwang, 2009). On the other hand, proline is not usually consumed in a rich nitrogen medium (Callejón et al., 2008). Besides, this amino acid could increase its concentration during cider fermentation by AAB (Valero et al., 2005).

In all the samples fermented with gluconic acid, asparagine-serine was the most abundant amino acid followed by arginine, glutamic acid, proline and ammonium ion. Gluconic fermentation did not modify the most essential amino acid from the strawberry purée substrate. Generally, tryptophan was the only amino acid that was significantly consumed for all AAB strains tested. Conversely, arginine increased wide its concentration in all

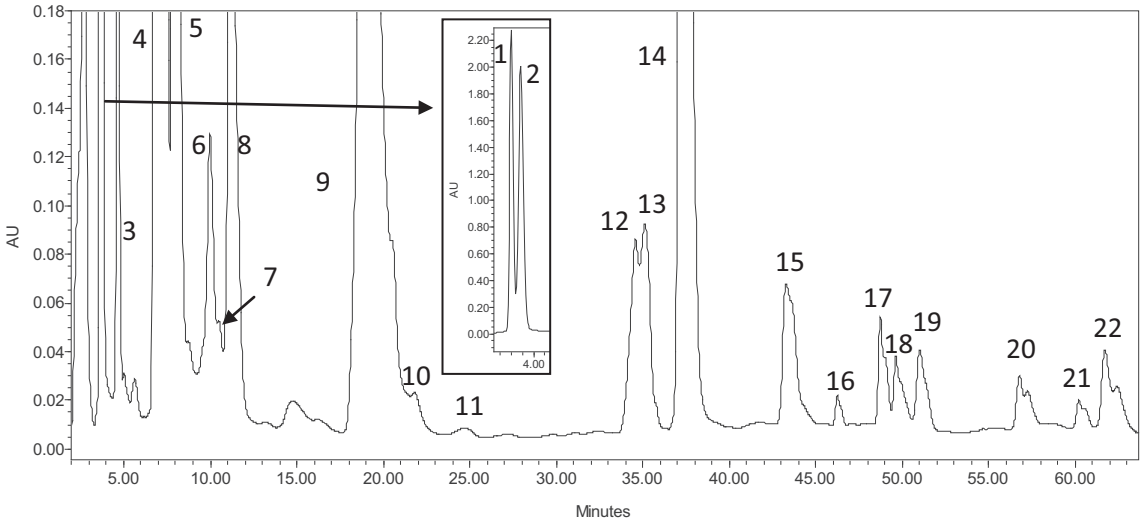

Fig. 1. Strawberry purée (substrate) HPLC Chromatogram at 280 nm. 1, aspartic acid; 2, glutamic acid; 3, internal standard; 4, asparagine-serine; 5, glutamine; 6, histidine; 7, glycine; 8, threonine; 9, alanine; 10, arginine; 11, γ-aminobutiric acid; 12, proline; 13, tyrosine; 14, ammonium ion; 15, valine; 16, methionine; 17, isoleucine; 18, tryptophan; 19, leucine; 20, phenylalanine; 21, ornithine; 22, lysine.

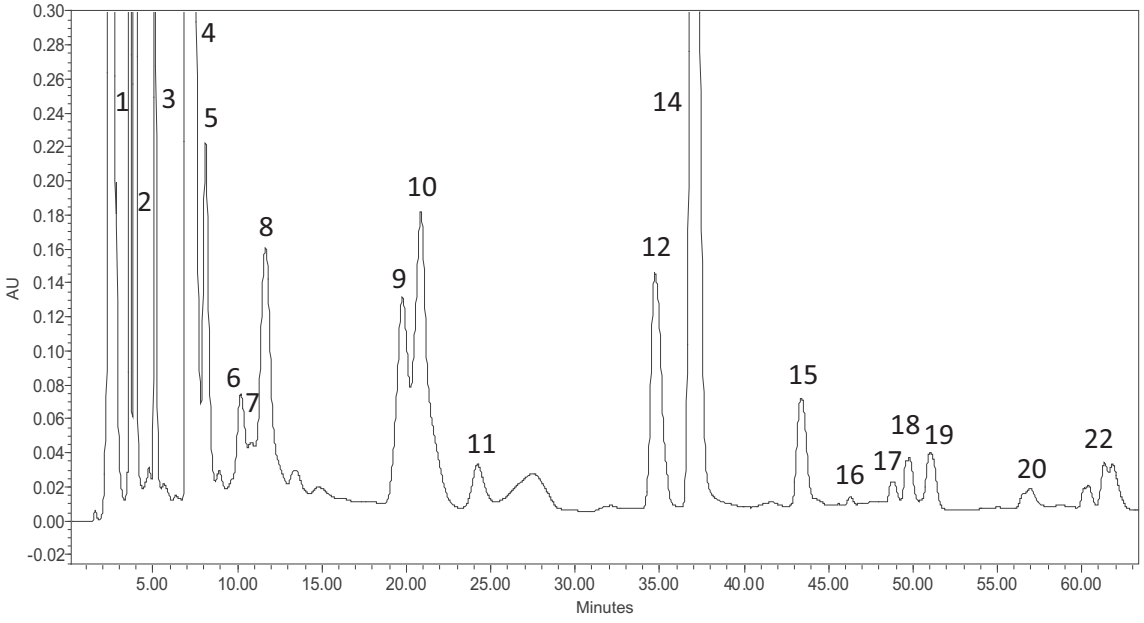

Fig. 2. *Gluconobacter japonicus* strain HPLC Chromatogram at 280 nm. 1, aspartic acid; 2, glutamic acid; 3, internal standard; 4, asparagine-serine; 5, glutamine; 6, histidine; 7, glycine; 8, threonine; 9, alanine; 10, arginine; 11, γ-aminobutiric acid; 12, proline; 14, ammonium ion; 15, valine; 16, methionine; 17, isoleucine; 18, tryptophan; 19, leucine; 20, phenylalanine; 22, lysine.

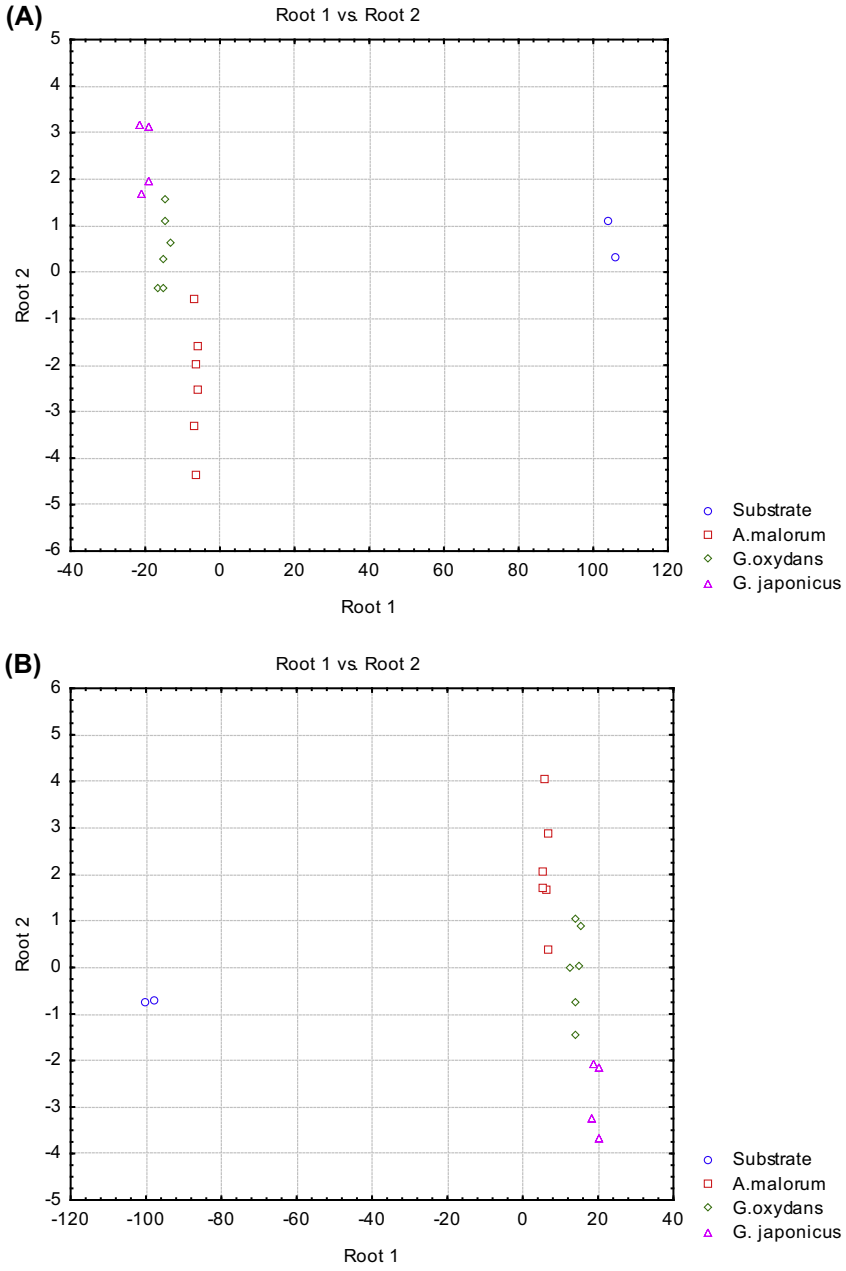

Fig. 3. The scatterplot of canonical scores of standard (A) and forward stepwise (B) analysis.

fermentations (Table 4). This is important because it is an essential amino acid to fetus, infant and adults with diseases such as endothelial dysfunction, cystic fibrosis or sickle cell disease vasculopathy (Wu, 2009; Wu et al., 2009).

Additionally, gluconic fermentation preserves polyphenols, which exert a bioactive effect and play an important role in the sensory properties (Álvarez-Fernández, Hornedo-Ortega, Cerezo, Troncoso, & García-Parrilla, 2014b).

Biogenic amines were not detected in the substrates or beverages. This was expected since according to Cipolla, Havouis, and Moulinoux (2010) strawberries were included in the group of food containing less than 100 nmol/g/ml of polyamines. Moreover, biogenic amines of endogenous origin are found in low concentrations (Önal, 2007). During fermentative processes, especially by lactic acid bacteria, the concentration of biogenic amines increases significantly (Landete, Ferrer, & Pardo, 2007). However, the non-presence of biogenic amines in the gluconic fermentations may be due to the fact that AAB are not able to produce these nitrogenous compounds (Landete et al., 2007).

Although amino acids and ammonium showed a similar trend during all gluconic fermentations, we observed some differences, depending on the bacteria involved in the process. Thus, *G. japonicus* strains were the one that showed the highest consumption of amino acids. In fact, significant differences in the amino acid profile were observed between *G. japonicus* strain and the other two strains. Glutamine, histidine, valine, ornithine, glutamic acid and isoleucine were consumed by the *G. japonicus* strain in a higher proportion than by the rest of the strains during fermentation. According to these results, we could conclude that *G. japonicus* strain showed the greatest activity during fermentation. This strain demonstrated the fastest glucose consumption, as no glucose was present in the medium after 10 days of fermentation.

In order to explore differences between the strains, multivariate statistical analysis was applied. First, we performed a principal

component analysis (PCA). We selected the most significant variables to gather different samples using the variable contribution obtained in PCA. Hence, Factor 1 includes glutamine, alanine, arginine and proline, and Factor 2 includes glutamic acid, asparagine-serine, isoleucine, leucine and phenylalanine. Afterwards, a linear discriminant analysis (LDA) was performed to evaluate whether the profile of amino acids and ammonium ion were different enough to distinguish the samples analyzed in this study based on the substrate and the AAB strain. For this purpose, we selected the variables mentioned above in Factor 1 and 2. Both standard and forward stepwise analyses were performed in LDA and the AAB strain was the grouping variable. The classification matrix was 100% for the standard and the forward stepwise analysis. However, while the standard analysis considered all these variables, the forward stepwise analysis did not select glutamic acid or phenylalanine. The scatterplot of the canonical scores of the standard and forward stepwise analyses are shown in Fig. 3. As it can be observed, the selected variables were able to clearly separate the substrate from the gluconic acid products. However, although the final products showed a similar amino acid profile, the LDA was able to group the samples from the gluconic acid fermented beverages according to the strain involved in the fermentation process.

4. Conclusions

A method for the determination of amino acid and biogenic amines in gluconic acid fermentation was successfully adapted, obtaining adequate values and demonstrating good linearity and precision, as well as low detection and quantification limits. Its utility for the routine analysis of amino acids and biogenic amines in this type of products has been shown.

The fermented products did not contain biogenic amines. The amino acid profile, specifically the concentrations of glutamine, alanine, arginine, proline, glutamic acid, asparagine–serine, isoleucine, leucine and phenylalanine allows the discrimination of the beverages according to the AAB strain responsible for the fermentation. Fermentation with *G. japonicus* resulted in major amino acid concentration changes.

Acknowledgments

We wish to thank the Ministry of Science and Innovation of the Spanish Government for its financial support through projects AGL2010-22152-C03-01 and AGL2010-22152-C03-02.

References

Álvarez-Fernández, M. A., Hornedo-Ortega, R., Cerezo, A. B., Troncoso, A. M., & García-Parrilla, M. C. (2014a). Effects of the strawberry (*Fragaria ananassa*) purée elaboration process on non-anthocyanin phenolic composition and antioxidant activity. *Food Chemistry, 164*, 104–112.

Álvarez-Fernández, M. A., Hornedo-Ortega, R., Cerezo, A. B., Troncoso, A. M., & García-Parrilla, M. C. (2014b). Non-anthocyanin phenolic compounds and antioxidant activity of beverages obtained by gluconic fermentation of strawberry. *Innovative Food Science & Emerging Technologies, 26*, 469–481.

Ancín-Azpilicueta, C., González-Marco, A., & Jiménez-Moreno, N. (2008). Current knowledge about the presence of amines in wine. *Critical Reviews in Food Science and Nutrition, 48*, 257–275.

Peer Verified Methods Program, Manual on Policies and Procedures, (1993). Arlington, VA: AOAC.

Arias-Gil, M., Garde-Cerdán, T., & Ancín-Azpilicueta, C. (2007). Influence of addition of ammonium and different amino acid concentrations on nitrogen metabolism in spontaneous must fermentation. *Food Chemistry, 103*(4), 1312–1318.

Callejón, R., Tesfaye, W., Torija, M., Mas, A., Troncoso, A., & Morales, M. (2008). HPLC determination of amino acids with AQC derivatization in vinegars along submerged and surface acetifications and its relation to the microbiota. *European Food Research and Technology, 227*(1), 93–102.

Callejón, R., Troncoso, A., & Morales, M. (2010). Determination of amino acids in grape-derived products: a review. *Talanta, 81*(4), 1143–1152.

Carrasco, P., & Pérez-Ortín, J. E. (2003). Arginase activity is a useful marker of nitrogen limitation during alcoholic fermentations. *Systematic and Applied Microbiology, 26*(3), 471–479.

Cerezo, A. B., Cuevas, E., Winterhalter, P., Garcia-Parrilla, M. C., & Troncoso, A. M. (2010). Isolation, identification, and antioxidant activity of anthocyanin compounds in camarosa strawberry. *Food Chemistry, 123*(3), 574–582.

Cipolla, B. G., Havouis, R., & Moulinoux, J. (2010). Polyamine reduced diet (PRD) nutrition therapy in hormone refractory prostate cancer patients. *Biomedicine & Pharmacotherapy, 64*(5), 363–368.

Deppenmeier, U., Hoffmeister, M., & Prust, C. (2002). Biochemistry and biotechnological applications of *Gluconobacter* strains. *Applied Microbiology and Biotechnology, 60*(3), 233–242.

Deppenmeier, U., & Ehrenreich, A. (2009). Physiology of acetic acid bacteria in light of the genome sequence of *Gluconobacter oxydans*. *Journal of Molecular Microbiology and Biotechnology, 16*(1–2), 69–80.

European Food Safety Authority (EFSA) Panel on Biological Hazards (BIOHAZ) (2011). Scientific Opinion on risk based control of biogenic amine formation in fermented foods. *EFSA Journal, 9*(10), 2393.

Fügel, R., Carle, R., & Schieber, A. (2005). Quality and authenticity control of fruit purées, fruit preparations and jams—A review. *Trends in Food Science & Technology, 16*(10), 433–441.

Gómez-Alonso, S., Hermosín-Gutiérrez, I., & García-Romero, E. (2007). Simultaneous HPLC analysis of biogenic amines, amino acids, and ammonium ion as aminoenone derivatives in wine and beer samples. *Journal of Agricultural and Food Chemistry, 55*(3), 608–613.

Gupta, A., Singh, V. K., Qazi, G., & Kumar, A. (2001). *Gluconobacter oxydans*: its biotechnological applications. *Journal of Agricultural and Food Chemistry, 3*(3), 445–456.

Hancock, J., Sjulin, T., & Lobos, G. (2008). Strawberries. *Temperate Fruit Crop Breeding*. Springer, pp. 393–437.

Hermosín, I., Chicón, R. M., & Dolores Cabezudo, M. (2003). Free amino acid composition and botanical origin of honey. *Food Chemistry, 83*(2), 263–268.

Hernández-Orte, P., Ibarz, M., Cacho, J., & Ferreira, V. (2003). Amino acid determination in grape juices and wines by HPLC using a modification of the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) method. *Chromatographia, 58*(1–2), 29–35.

Hidalgo, C., Torija, M., Mas, A., & Mateo, E. (2013). Effect of inoculation on strawberry fermentation and acetification processes using native strains of yeast and acetic acid bacteria. *Food Microbiology, 34*(1), 88–94.

Hui, Y., Barta, J., Canor, M., Gusek, T., Sidhu, J., & Sinha, N. (2006). *Handbook of Fruits and Fruit Processing*. Iowa: Blackwell Publishing.

Joubert, J., Bayens, W., & De Ley, J. (1961). The catabolism of amino acids by acetic acid bacteria. *Antonie Van Leeuwenhoek, 27*(1), 151–160.

Kim, J. Y., Lee, M. Y., Ji, G. E., Lee, Y. S., & Hwang, K. T. (2009). Production of γ-aminobutyric acid in black raspberry juice during fermentation by *Lactobacillus brevis* GABA100. *International Journal of Food Microbiology, 130*(1), 12–16.

Kirschbaum, J., Rebscher, K., & Brückner, H. (1999). Determination of Biogenic Amines in Fermented Beverages and Vinegars by Pre-column Derivatization with para-Nitrobenzyloxycarbonyl Chloride (PNZ-Cl) and Reversed-Phase LC. *Chromatographia, 49*, 117–124.

Landete, J. M., Ferrer, S., & Pardo, I. (2007). Biogenic amine production by lactic acid bacteria, acetic bacteria and yeast isolated from wine. *Food Control, 18*(12), 1569–1574.

Leigh, J. A., & Dodsworth, J. A. (2007). Nitrogen regulation in bacteria and archaea. *The Annual Review of Microbiology, 61*, 349–377.

Mandrioli, R., Mercolini, L., & Raggi, M. A. (2013). Recent trends in the analysis of amino acids in fruits and derived foodstuffs. *Analytical and Bioanalytical Chemistry, 405*(25), 7941–7956.

Merrick, M. J., & Edwards, R. A. (1995). Nitrogen control in bacteria. *Microbiological Reviews, 59*(4), 604–622.

Moing, A., Renaud, C., Gaudillère, M., Raymond, P., Roudeillac, P., & Denoyes-Rothan, B. (2001). Biochemical changes during fruit development of four strawberry cultivars. *Journal of the American Society for Horticultural Science, 126*(4), 394–403.

Önal, A. (2007). A review: current analytical methods for the determination of biogenic amines in foods. *Food Chemistry, 103*(4), 1475–1486.

Peña-Gallego, A., Hernández-Orte, P., Cacho, J., & Ferreira, V. (2009). Biogenic amine determination in wines using solid-phase extraction: a comparative study. *Journal of Chromatography A, 1216*(15), 3398–3401.

Peña-Gallego, A., Hernández-Orte, P., Cacho, J., & Ferreira, V. (2012). High-performance liquid chromatography analysis of amines in must and wine: a review. *Food Reviews International, 28*(1), 71–96.

Perez, A. G., Rios, J. J., Sanz, C., & Olias, J. M. (1992). Aroma components and free amino acids in strawberry variety chandler during ripening. *Journal of Agricultural and Food Chemistry, 40*(11), 2232–2235.

Ramachandran, S., Fontanille, P., Pandey, A., & Larroche, C. (2006). Gluconic acid: properties, applications and microbial production. *Food Technology and Biotechnology, 44*(2), 185–195.

Sánchez, S., & Demain, A. L. (2002). Metabolic regulation of fermentation processes. *Enzyme and Microbial Technology, 31*(7), 895–906.

Singh, O. V., & Kumar, R. (2007). Biotechnological production of gluconic acid: future implications. *Applied Microbiology and Biotechnology, 75*(4), 713–722.

StatSoft Inc. (2004). STATISTICA (data analysis software system), version 7. <www.statsoft.com>.

Stürtz, M., Cerezo, A. B., Cantos-Villar, E., & Garcia-Parrilla, M. C. (2011). Determination of the melatonin content of different varieties of tomatoes

(*Lycopersicon esculentum*) and strawberries (*Fragaria ananassa*). *Food Chemistry, 127*(3), 1329–1334.

ten Brink, B., Damink, C., Joosten, H., & Huis In't Veld, J. (1990). Occurrence and formation of biologically active amines in foods. *International Journal of Food Microbiology, 11*(1), 73–84.

Ubeda, C., Callejón, R., Hidalgo, C., Torija, M., Troncoso, A., & Morales, M. (2013). Employment of different processes for the production of strawberry vinegars: effects on antioxidant activity, total phenols and monomeric anthocyanins. *LWT-Food Science and Technology, 52*(2), 139–145.

Valero, E., Berlanga, T. M., Roldan, P. M., Jimenez, C., García, I., & Mauricio, J. C. (2005). Free amino acids and volatile compounds in vinegars obtained from different types of substrate. *Journal of the Science of Food and Agriculture, 85*(4), 603–608.

Wu, G. (2009). Amino acids: metabolism, functions, and nutrition. *Amino Acids, 37*(1), 1–17.

Wu, G., Bazer, F. W., Davis, T. A., Kim, S. W., Li, P., Rhoads, J. M., et al. (2009). Arginine metabolism and nutrition in growth, health and disease. *Amino Acids, 37*(1), 153–168.

Artículo 3

Effect of gluconic acid submerged fermentation of strawberry purée on amino acids and biogenic amines profile.

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Journal of Food Processing and Preservation
41 (2017)

Q3; 96/130 (JCR-ISI, Food Science & Technology, 2016)
Impact Factor: 0.791

EFFECT OF GLUCONIC ACID SUBMERGED FERMENTATION OF STRAWBERRY PURÉE ON AMINO ACIDS AND BIOGENIC AMINES PROFILE

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Received for Publication September 8, 2015
Accepted for Publication December 8, 2015

doi:10.1111/jfpp.12787

ABSTRACT

Gluconic fermentation of fruit substrates is an innovative strategy to elaborate nonalcoholic beverages which has not been studied so far. Futhermore, it prevents food waste of perishable products like strawberries. Amino acids, biogenic amines and ammonium ion were evaluated after the fermentation of strawberry purée using *Gluconobacter japonicus* in submerged culture. The process was monitored with the determination of pH, total acidity, glucose, fructose and gluconic acid concentrations. The determination of nitrogen compounds was accomplished using a HPLC–UV method involving diethyl ethoxymethylenemalonate (DEEMM) as the derivatization agent. Most amino acids did not present significant changes, except for lysine and histidine, which increased their concentration in the final product. However, the principal component analysis was able to group the initial and final samples in different sets using the amino acids concentration as variables. Biogenic amines were not detected in the final samples, thus ensuring the safety of this product for human consumption.

PRACTICAL APPLICATIONS

Because of the high level of competitiveness, the success of a new product requires the use of a raw material different from the traditionally used ones, and the application of innovative techniques of production. Strawberry is a fruit prized for its aroma and flavor. In addition, Spain is the fifth strawberry producer in the world, and consequently there are surpluses of second quality strawberries. This second quality fruit is generally discarded, involving large economic losses. These facts make strawberry a good candidate to be used as raw material for the production of fruit derived products. Hence, gluconic acid fermentation is an innovative way of transforming surplus strawberries into beverages which do not present glucose, as it is transformed into gluconic acid whilst keeping fructose as sweetener. Hence, the final product could be the principal substract to elaborate a new beverage which could be consumed by diabetics. Gluconic acid submerged fermentations of strawberry purée were carried out with *Gluconobacter japonicus*. Additionally, the amount of biogenic amines was assessed for food safety purposes.

INTRODUCTION

Gluconobacter has been employed to obtain several compounds in biotechnological industries, such as production of D-gluconic acid, 5-keto-gluconic acid and 2-ketogluconic acid from D-glucose. Moreover, this genus could synthesize L-sorbose from D-sorbitol and dihydroxyacetone from glycerol. In particular, gluconic acid production is one of its main applications (Gupta *et al.* 2001). Thus, about

60,000 tons of this compound is produced annually (Purane *et al.* 2012).

Gluconic acid is employed for pickling foods or cleaning aluminium cans. Glucono-δ-lactone is a precursor of synthesis pathway of gluconic acid that is used as an acidulant and flavoring agent. Besides, its salts are used in pharmaceutical industries, like calcium salt of gluconic acid for calcium therapy or iron salt of gluconic salt for the treatment of anemia. On the other hand, sodium gluconate is employed as an additive in cement production, detergent in bottle washing or to prevent iron deposits in textile industries (Gupta *et al.* 2001; Ramachandran *et al.* 2006; Singh and Kumar 2007).

Additionally, this acid is present in a wide number of foods such as apples, dates or grapes and honeybees (up 12 g/kg) (Mato *et al.* 1997; Ramachandran *et al.* 2006).

Moreover, gluconic acid is employed as a food additive. This acidity regulator is included on the approved list of additives (E 574) of Commission Regulation (EU) No 1130/2011 without limitation. Furthermore, U.S. Food and Drug Administration (FDA) included gluconic salt (sodium, calcium, copper, ferrous and manganese gluconate) and glucono-δ-lactone are generally recognized as safe (GRAS) in the list (FDA 2014).

Besides, gluconic acid could be present in fermented products such as wine, beer, cider and vinegar (Gupta *et al.* 2001; Ramachandran *et al.* 2006).

Moreover, new beverages from fruit substrate containing a certain amount of gluconic acid are being developed. These products could be an interesting option for using fruit of second quality as well as meeting the demand of new products that could maintain the healthy properties of fruits.

Gluconic acid and its counterparts have usually been produced industrially by fermentation. Even though *Aspergillus niger* has been widely employed for gluconic acid production, it is not until nowadays that *Gluconobacter oxydans* has been used (Ramachandran *et al.* 2006).

G. oxydans, G. japonicus and *Acetobacter malorum* were tested to ferment strawberry in a previous work. Among them, *G. japonicus* proved to convert glucose into gluconic acid more efficiently (Ordóñez *et al.* 2015). Some changes were observed in the amino acid profile in gluconic acid fermentations of strawberry purée using surface cultures of different species of acetic acid bacteria. However, submerged culture systems provide a much faster alternative and it is more interesting in an industrial scale (Mas *et al.* 2014).

Regulations (EC) No 258/97 require a safety evaluation when introducing a new food or food processing method. As gluconic acid fermentation has previously been scarcely used, it is advisable to evaluate if these substances could be produced complying with safety concerns.

In this context, biogenic amines can be produced from amino acids by decarboxylation. Biogenic amines are found at low concentrations in raw products, but its amount could be much higher in fermented products, such as red wine or cheese (Ancín-Azpilicueta *et al.* 2008). The consumption of high amounts of biogenic amines in food could have toxicological effects, especially for sensitive consumers (Ten Brink *et al.* 1990). These compounds may be a good indicator of contamination in the product (Callejón *et al.* 2010).

In this context, the aim of this study was to study the differences in amino acids, biogenic amines and ammonium ion profile in gluconic acid submerged fermentations of strawberry purée; and to check that biogenic amines are not produced and the products are safe.

MATERIALS AND METHODS

Reagents and Standards

Most of the amino acid standards were purchased from Fluka (Buchs, Switzerland). The aspartic acid, glutamic acid, histidine, alanine, lysine, γ-aminobutiric acid (GABA), biogenic amines, ammonium sulphate, diethyl ethoxymethylenemalonate, acetic acid glacial, boric acid, 2-aminoadipic acid (internal standard) and sodium azide were supplied by Sigma-Aldrich (Steinheim, Germany). The glycine, ornithine, methanol (HPLC grade) and acetonitrile (HPLC grade) were acquired from Merck (Darmstadt, Germany). The sodium acetate and sodium hydroxide were obtained from Panreac (Castellar del Vallès, Barcelona). The ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

The stock standard solutions were prepared individually by dissolving the pure compounds in HCl 0.1 N. The calibration solutions were prepared by diluting the stock standard solutions with water.

Samples

Strawberry purée industrially produced by Hudisa, S.A. (Lepe, Spain) was used as substrate. The purée (2012 harvest) was previously pasteurized and stored at 0–4C containing 34.00 ± 1.25 g sugars per liter (45% glucose and 55% fructose).

A *G. japonicus* strain (CECT 8443), isolated from grape must (Navarro *et al.* 2013), was used as a starter for the submerged fermentation process. The inoculum was prepared by seeding in 250 mL Erlenmeyer flasks containing 125 mL of GYP medium (5% (w/v) glucose, 1% (w/v) yeast extract and 2% (w/v) bacteriological peptone) previously autoclaved at 120C for 15 min. After shaking in an incubator at 29C at 150 rpm for 24 h, each flask was supplied with

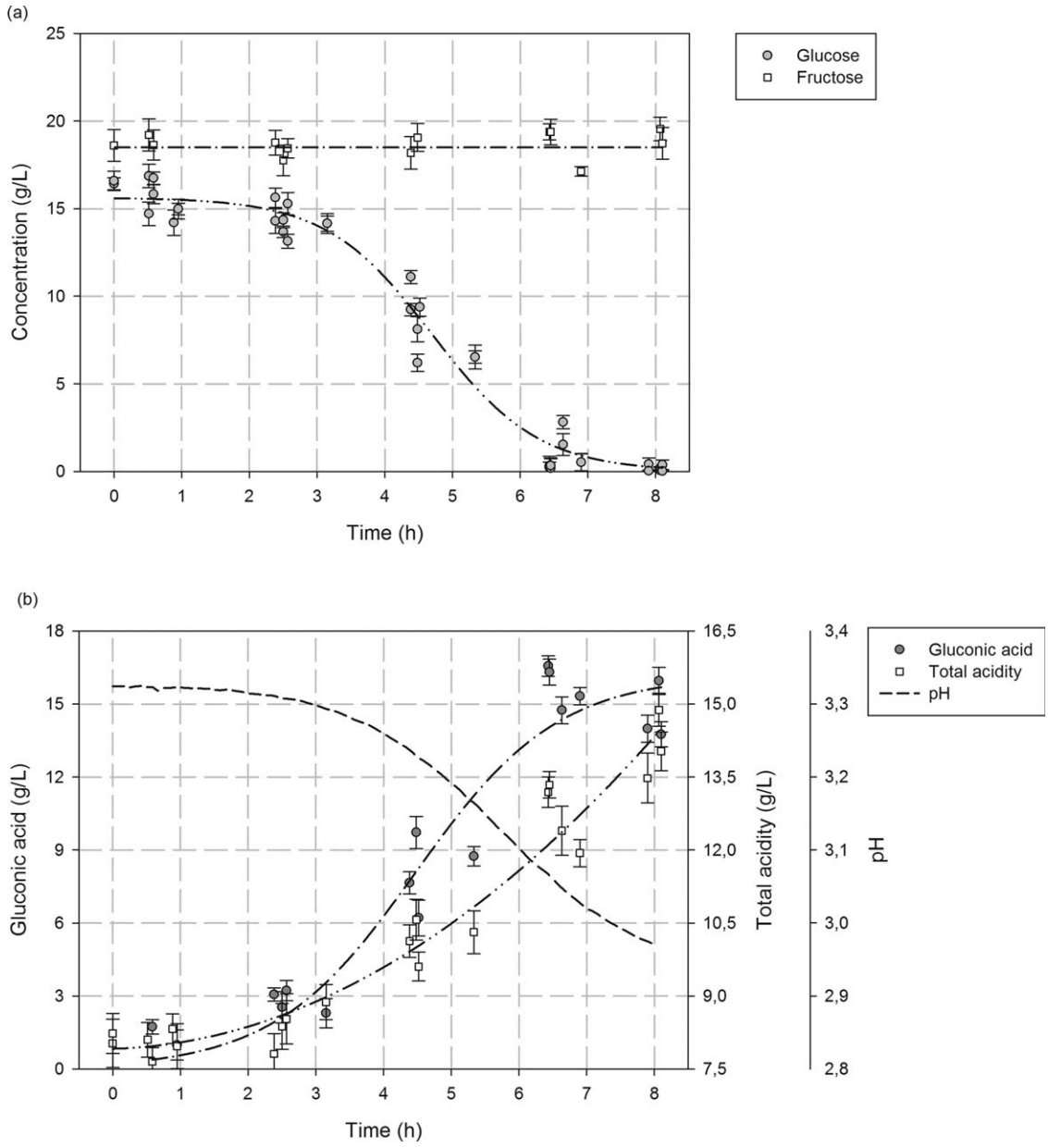

FIG. 1. VARIATION OF GLUCOSE, FRUCTOSE, GLUCONIC ACID AND TOTAL ACIDITY CONCENTRATION AND PH DURING GLUCONIC FERMENTATION BY G. japonicus CECT 8443

125 mL of sterilized strawberry purée under the same incubation conditions for additional 24 h.

Fermentation runs were conducted in a 5 L Biostat® fermenter equipped with pH, agitation, oxygen and temperature controls, operating in a batch mode. The operational variables were set as follows for all tests: substrate volume, 3 L; agitation, 500 rpm; temperature, 29C; and dissolved oxygen, 20%. Only the pH of the medium was allowed to evolve freely throughout the process. Totally, four fermentation cycles were performed.

Samples were taken at the beginning of fermentation, just after the inoculum addition (initial samples), and at the end of fermentation, when glucose was totally exhausted (final samples). The samples were pasteurized by heating at 70–80C for 15 min and then rapidly cooled and frozen until analysis.

Sugars and gluconic acid contents were quantified with the following enzyme kits from Megazyme®: K-GLUC 07/11 for glucose, K-FRUGL 12/12 for fructose and K-GATE 12/12 for gluconic acid. Additional information about the kits can be found at the manufacturer's website (www.megazyme.com). Total acidity was analyzed by acid–base titration. All determinations were performed at least in triplicate. The ensuing standard deviations are shown in Fig. 1A–C.

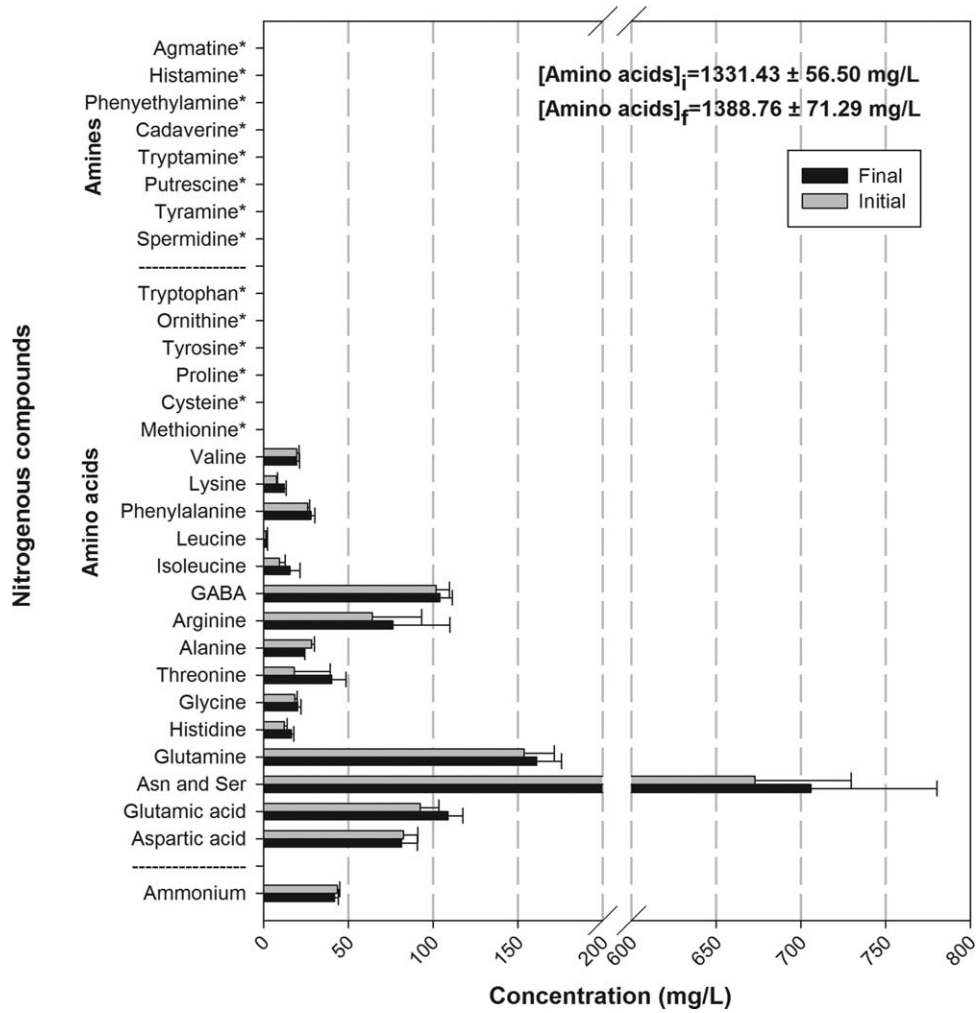

FIG. 2. AMINO ACIDS, BIOGENIC AMINES AND AMMONIUM ION CONCENTRATION DURING GLUCONIC FERMENTATION OF STRAWBERRY PURÉE. THE GLOBAL AMINO ACID BOTH AT THE BEGINNING AND AT THE END OF THE PROCESS WERE CALCULATED WITHOUT CONSIDERING AMMONIUM ION CONTENT

Sample Preparation

First, we centrifuged 2 mL of sample at 6000 rpm for 15 min (Eppendorf centrifuge 5415R, Hamburg, Germany). For amino acids, biogenic amines and ammonium ion derivatization, diethyl ethoxymethylenemalonate (DEEMM) was used. The analyses of these compounds were performed using the method described by Gómez-Alonso *et al.* (2007). All samples were filtered through a membrane filter with a mean pore size of 0.45 μm (Millipore) prior to use.

Equipment

HPLC analysis was carried out in a Waters equipment consisting of a Waters 717 autosampler injector and a Waters 1525 Binary HPLC pump system controller connected to a Waters 996 photodiode array detector. Data treatment was performed in a Waters Millennium data station. The column consisted of a LiChroCART® 250-4 LiChrospher® 100 RP-18 (5 μm, 250 × 4.6 mm) from Merck (Darmstadt, Germany) and a 4.0 × 3.0 mm guard column from Analytical Phenomenex (Torrance, CA, USA). The column was thermostatized at 45C in a column header module controlled by Waters TCM HPLC Temperature Controller. The gradient program used was employed by Ordóñez *et al.* (2015).

Statistical Analysis

All statistical analyses were performed by means of Statistica software (StatSoft, 2004). One-way ANOVA was performed to evaluate significant differences between types of samples (significance levels $P < 0.05$). Principal Component Analysis (PCA) was employed to evaluate whether the profiles of

amino acids and biogenic amines were different enough to distinguish between initial and final samples.

RESULTS AND DISCUSSION

Chemical Changes During Gluconic Fermentation

Figure 1 shows the variation of the glucose, fructose, gluconic acid and total acidity concentrations and the pH of the medium during the fermentation. As can be seen, glucose was completely consumed after the first 8 h while fructose remained stable until the end of the fermentation process (Fig. 1a). At the same time, gluconic acid increased in inverse proportion to glucose consumption, with a final concentration of 15.69 ± 0.52 g/L (Fig. 1b). Furthermore, differences in the total acidity and the pH of the medium were also observed following the gluconic acid production: the former increased up to 6.47 ± 0.64 g/L while the later dropped to an average of 0.35 ± 0.03 units of pH (Fig. 1b).

The quick biotransformation and the high yield obtained for gluconic acid production, 92.43%, suggest that the strain of *G. japonicus* used was perfectly adapted to the complex medium. Among other reasons, this was because of its ability to grow under acidic conditions (Malimas *et al.* 2009), as in the case of strawberry purée. Equally noticeable is the *G. japonicus* capacity to convert glucose from strawberry with high selectivity, preserving the fructose content and thereby some of the original sweetness of the fruit. These results were consistent with previous works in this field (Sainz *et al.* 2012; Álvarez-Fernández *et al.* 2014).

Evolution of Nitrogen Compounds Along Fermentation

In order to study the variance of the major nitrogenous compounds of fermented strawberry purée, the biogenic amines, amino acids and ammonium ion contents were determined both at the beginning and at the end of gluconic acid fermentation. Figure 2 shows the average values and standard deviations for 31 nitrogenous compounds, concluding that there were no significant differences between initial and final samples, with the exception of lysine and histidine. Coinciding with previous works (Moing *et al.* 2001; Callejón *et al.* 2015), asparagine and serine were the major amino acids in strawberry purée, followed by glutamine, glutamic and aspartic acids, and GABA. From a global point of view, no significant differences were detected in the total amino acid content between strawberry purée (19.91 ± 0.93 mM N) and the fermented product (20.43 ± 1.21 mM N).

Conversely, in previous studies after gluconic acid fermentation of strawberry purée using a surface culture of AAB, a decrease of amino acids was observed (Ordóñez *et al.* 2015). However, Callejón *et al.* (2008) stated that AAB in submerged acetifications had a catalytic activity while surface fermentation had an active metabolism and growth. Besides, surface and submerged cultures showed a different duration of the fermentation processes. Indeed, fermentation with submerged cultures took 8 h to consume the glucose of the substrate, while surface cultures took 10 days (Ordóñez *et al.* 2015). In addition, Nie *et al.* (2013) observed that the content of amino acid nitrogen increased after acetic acid fermentation, possibly because of microbial lysis. This could be because of the heat treatment (pasteurization) applied after the fermentation, that causes lysis of the bacteria.

Therefore, these three facts show that catalytic activity, duration of fermentation and pasteurization could explain the results obtained during the submerged process.

Another point to be considered is the total acidity evolution during gluconic acid fermentation (Fig. 1c). As can be seen, *G. japonicus* activity caused the acidification of the medium, which could favor the extraction of amino acids from plant cells (Zhang *et al.* 2014).

Therefore, the final balance on the amino acids content is a quite complex issue depending on multiple aspects.

Similarly, Cerrillo *et al.* (2015) showed that the initial concentration of amino acids was similar after alcoholic fermentation of orange juice had finished. Besides, Álvarez-Fernández *et al.* (2014) observed that bioactive compounds (polyphenols) and antioxidant activity were maintained after the gluconic acid fermentation of strawberry purée. Therefore, this fermentation allows to maintain the nutritional value of the substrate in the final product.

Principal Component Analysis (PCA)

In order to explore differences between initial and final samples under study, a principal component analysis (PCA) was performed considering the amino acids and ammonium ion as variables. However, it was observed that there was not a pattern to separate initial and final fermented samples.

Thus, we selected the most contributing variables to the variance of the data matrix to group different samples. Hence, Factor 1 includes lysine, phenylalanine, glutamic acid, isoleucine and histidine, and Factor 2 includes aspartic acid, leucine, alanine and ammonium ion.

Afterwards, we applied PCA again, selecting the variables mentioned above in Factors 1 and 2. It was found that the two principal components accounted for 84.8% of the cumulative variance. Figure 3 shows the scores samples and loadings variables plotted using the first two principal components. As we can see, the selected variables were able to clearly separate initial and final fermentation. Although the final products showed a similar amino acid profile, the PCA was able to group the samples.

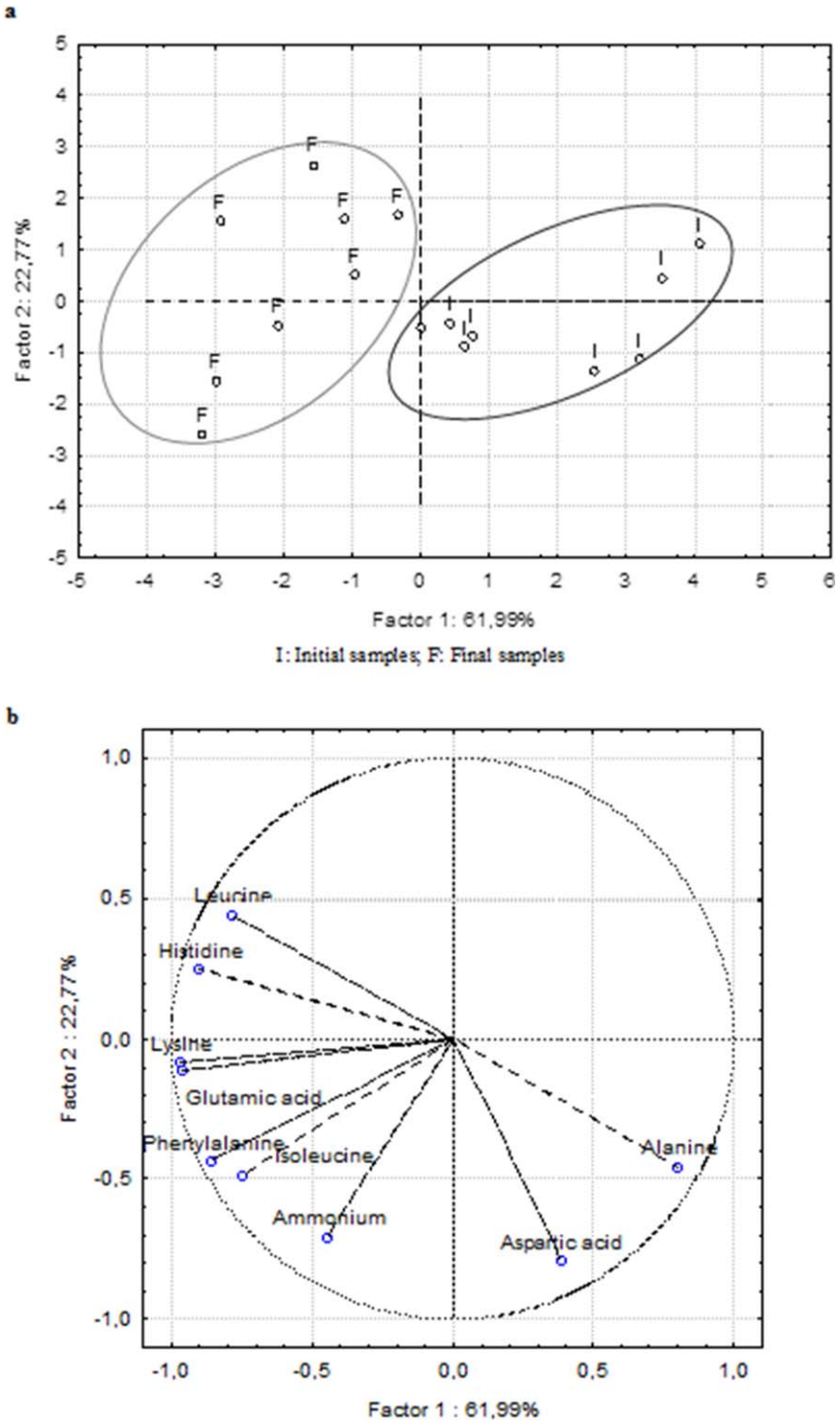

FIG. 3. (A) GRAPHS OF SCORES OF INITIAL AND FINAL SAMPLES AND (B) LOADINGS OF VARIABLES USING FIRST TWO PRINCIPAL COMPONENTS

PC1 seems to separate initial and final fermentation samples. Thus, initial fermentation samples were placed on the right side of the graph, whilst final fermented were on the left. According to the loadings, initial samples were more related to alanine and aspartic acid (Fig. 2). We could observe that these amino acids had a trend to decrease their concentration, although the differences between initial and final fermented samples were not significant. These amino

acids are good nitrogen sources (Arias-Gil *et al*. 2007) and they could be consumed by AAB.

On the other hand, final samples were more linked to those nitrogen compounds that showed an increase during the fermentations process, mainly histidine and lysine. Lysine had been determined in a higher concentration in final alcoholic fermentations of strawberry than in substrate in a previous work (Callejón *et al*. 2015). Besides, Cerrillo *et al*. (2015) observed an increase of lysine in alcoholic fermentation, whilst histidine did not have a significant change in its concentration. The rest of variables associated to final samples according to the loadings, such as glutamic acid, isoleucine and phenylalanine, had a trend to rise after gluconic acid fermentation, although their concentrations did not have a significant increase (Fig. 2).

Biogenic amines were not detected in the initial substrates or the fermented. Regarding AAB, it has been observed that these bacteria are not able to produce biogenic amines during acetic fermentation (Landete *et al*. 2007). Thus, these fermented products are safe for human consumption in terms of biogenic amines content.

CONCLUSIONS

Submerged fermentation of strawberry purée did not produced significant changes on the amino acids profile, allowing to maintain its initial nutritional value. Among the amino acids determined highlight lysine and histidine, since presented a significant increase in their concentrations from the initial to the final samples.

At the beginning as well as at the end of the gluconic acid fermentations biogenic amines were not synthetized. Thus, we can state that these fermented can be useful for the elaboration of consumer products.

The amino acid and ammonium ion profile, specifically lysine, phenylalanine, glutamic acid, isoleucine, histidine, aspartic acid, leucine, alanine and ammonium ion, allow to group initial and final samples of the fermentations.

ACKNOWLEDGMENTS

We wish to thank the Ministry of Science and Innovation of the Spanish Government for its financial support through projects AGL2010-22152-C03-01 and AGL2010-22152-C03-03. We would also like to thank HUDISA Desarrollo Industrial S.A., in Lepe, Spain, for providing the strawberry purée.

REFERENCES

ÁLVAREZ-FERNÁNDEZ, M.A., HORNEDO-ORTEGA, R., CEREZO, A.B., TRONCOSO, A.M. and GARCÍA-PARRILLA, M.C. 2014. Non-anthocyanin phenolic compounds and antioxidant activity of beverages obtained by gluconic fermentation of strawberry. Innov. Food Sci. Emerg. Technol. *26*, 469–481.

ANCÍN-AZPILICUETA, C., GONZÁLEZ-MARCO, A. and JIMÉNEZ-MORENO, N. 2008. Current knowledge about the presence of amines in wine. Crit. Rev. Food Sci. Nutr. *48*, 257–275.

ARIAS-GIL, M., GARDE-CERDÁN, T. and ANCÍN-AZPILICUETA, C. 2007. Influence of addition of ammonium and different amino acid concentrations on nitrogen metabolism in spontaneous must fermentation. Food Chem. *103*, 1312–1318.

CALLEJÓN, R.M., TESFAYE, W., TORIJA, M.J., MAS, A., TRONCOSO, A.M. and MORALES, M.L. 2008. HPLC determination of amino acids with AQC derivatization in vinegars along submerged and surface acetifications and its relation to the microbiota. Eur. Food Res. Technol. *227*, 93–102.

CALLEJÓN, R., TRONCOSO, A. and MORALES, M. 2010. Determination of amino acids in grape-derived products: A review. Talanta *81*, 1143–1152.

CALLEJÓN, R.M., UBEDA, C., HIDALGO, C., MAS, A., TRONCOSO, A.M. and MORALES, M.L. 2014. Changes on free amino acids during the alcoholic fermentation of strawberry and persimmon. Int. J. Food Sci. Technol. *50*, 48–54.

CERRILLO, I., FERNÁNDEZ-PACHÓN, M.S., COLLADO-GONZÁLEZ, J., ESCUDERO-LÓPEZ, B., BERNÁ, G., HERRERO-MARTÍN, G., MARTÍN, F., FERRERES, F. and GIL-IZQUIERDO, A. 2015. Effect of fermentation and subsequent pasteurization processes on amino acids composition of orange juice. Plant Food Hum. Nutr. *70*, 153–159.

COMMISSION REGULATION (EU) 2011. No 1130/2011 of 11 November 2011 amending Annex III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives by establishing a Union list of food additives approved for use in food additives, food enzymes, food flavourings and nutrients.

FDA 2014. Substances generally recognized as safe. Available from: http://www.fda.gov/food/ingredientspackaginglabeling/foodadditivesingredients/ucm091048.htm (accessed July 15, 2015).

GÓMEZ-ALONSO, S., HERMOSÍN-GUTIÉRREZ, I. and GARCÍA-ROMERO, E. 2007. Simultaneous HPLC analysis of biogenic amines, amino acids, and ammonium ion as aminoenone derivatives in wine and beer samples. J. Agric. Food Chem. *55*, 608–613.

GUPTA, A., SINGH, V.K., QAZI, G. and KUMAR, A. 2001. *Gluconobacter oxydans*: Its biotechnological applications. J. Agric. Food Chem. *3*, 445–456.

LANDETE, J.M., FERRER, S. and PARDO, I. 2007. Biogenic amine production by lactic acid bacteria, acetic bacteria and yeast isolated from wine. Food Control. *18*, 1569–1574.

MALIMAS, T., YUKPHAN, P., TAKAHASHI, M., MURAMATSU, Y., KANEYASU, M., POTACHAROEN, W.,

TANASUPAWAT, S., NAKAGAWA, Y., TANTICHAROEN, M. and YAMADA, Y. 2009. *Gluconobacter japonicus* sp. nov., an acetic acid bacterium in the *Alphaproteobacteria*. Int. J. Syst. Evol. Microbiol. *59*, 466–471.

MAS, A., TORIJA, M.J., GARCÍA-PARRILLA, M.D.C. and TRONCOSO, A.M. 2014. Acetic acid bacteria and the production and quality of wine vinegar. Sci. World J. *2014*, 6 pages.

MATO, I., HUIDOBRO, J.F., SÁNCHEZ, M.P., MUNIATEGUI, S., FERNÁNDEZ-MUIÑO, M.A. and SANCHO, M.T. 1997. Enzymatic determination of total D-gluconic acid in honey. J. Agric. Food Chem. *45*, 3550–3553.

MOING, A., RENAUD, C., GAUDILLÈRE, M., RAYMOND, P., ROUDEILLAC, P. and DENOYES-ROTHAN, B. 2001. Biochemical changes during fruit development of four strawberry cultivars. J. Am. Soc. Hortic. Sci. *126*, 394–403.

NAVARRO, D., MATEO, E., TORIJA, M.J. and MAS, A. 2013. Acetic acid bacteria in grape must. Acetic Acid Bact. *2*, 19–23.

NIE, Z., ZHENG, Y., WANG, M., HAN, Y., WANG, Y., LUO, J. and NIU, D. 2013. Exploring microbial succession and diversity during solid-state fermentation of Tianjin duliu mature vinegar. Bioresour. Technol. *148*, 325–333.

ORDÓÑEZ, J.L., SAINZ, F., CALLEJÓN, R.M., TRONCOSO, A.M., TORIJA, M.J. and GARCÍA-PARRILLA, M.C. 2015. Impact of gluconic fermentation of strawberry using acetic acid bacteria on amino acids and biogenic amines profile. Food Chem. *178*, 221–228.

PURANE, N.K., SHARMA, S.K., SALUNKHE, P.D., LABADE, D.S. and TONDLIKAR, M.M. 2012. Gluconic acid production from golden syrup by *Aspergillus niger* strain using semiautomatic stirred-tank fermenter. J. Microb. Biochem. Technol. *4*, 092–095.

RAMACHANDRAN, S., FONTANILLE, P., PANDEY, A. and LARROCHE, C. 2006. Gluconic acid: Properties, applications and microbial production. Food Technol. Biotechnol. *44*, 185–195.

REGULATION (EC) No 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients. Official Journal L. *43*(14/02), 0001–0006.

SAINZ, F., MATEO, E., MAS, A. and TORIJA, M.J. 2012. Gluconic acid production in strawberry by acetic acid bacteria. Acetic Acid Bact. *1*, 24.

STATSOFT, INC. 2004. STATISTICA (data analysis software system), version 7. www.statsoft.com.

SINGH, O.V. and KUMAR, R. 2007. Biotechnological production of gluconic acid: Future implications. Appl. Microbiol. Biotechnol. *75*, 713–722.

TEN BRINK, B., DAMINK, C., JOOSTEN, H. and HUIS IN'T VELD, J. 1990. Occurrence and formation of biologically active amines in foods. Int. J. Food Microbiol. *11*, 73–84.

ZHANG, H., WANG, Z.Y., YANG, X., ZHAO, H.T., ZHANG, Y.C., DONG, A.J., JING, J. AND WANG, J. 2014. Determination of free amino acids and 18 elements in freeze-dried strawberry and blueberry fruit using an amino acid analyzer and ICP-MS with micro-wave digestion. Food Chem. *147*, 189–194.

CAPÍTULO III

Resumen

Aunque en el capítulo anterior se valida y aplica un método para determinar aminas biógenas en productos fermentados, es importante disponer de métodos que sean más específicos y sensibles para poder determinar concentraciones más pequeñas y evitar la interferencias de otros compuestos, como los aminoácidos.

Así, este capítulo se centra en la validación y aplicación de un método cromatográfico para determinar aminas biógenas en productos fermentados (vino y vinagres). Para ello, las muestras fueron extraídas mediante una extracción en fase sólida (SPE) con un cartucho de resinas de modo mixto (Oasis MCX 1cc). Posteriormente, fueron derivatizadas con 6-aminoquinolil-N-hidroxisuccinimidil carbamato (AQC) como reactivo derivatizante precolumna y se analizaron con un cromatógrafo líquido acoplado a un detector de fluorescencia.

El método analítico fue validado exitosamente para la determinación de 9 aminas biógenas en vinagres, (histamina, tiramina, putrescina, cadaverina, agmatina, espermidina, espermina, metilamina y feniletilamina) en términos de selectividad, linealidad, sensibilidad (LD y LQ), precisión (repetibilidad y precisión intermedia) y recuperación de acuerdo con los criterios establecidos por AOAC (AOAC, 1993).

El método validado fue empleado para determinar aminas biógenas en diferentes tipos de vinagres, entre los que se encuentran 6 tipos de vino tinto, 4 de vino blanco, 3 de manzana y 3 balsámicos. Además se evaluaron 10 vinagres de vino de Jerez, divididos en 3 vinagres de Jerez, 3 vinagres de Jerez Reserva, 3 vinagres de Jerez Gran Reserva y 1 Pedro Ximénez. El contenido total de aminas biógenas en vinagres osciló entre 23.35 y 1445.2 μg/L. La metilamina y la feniletilamina no fueron detectadas en ningún vinagre. Por otro lado, putrescina fue la amina biógena que presentó mayores concentraciones en la mayoría de las muestras. De los vinagres analizados, los vinagres balsámicos y el vinagre "Pedro Ximénez" presentaron mayor concentración de aminas biógenas, mientras que las cantidades más baja fueron obtenidas en los vinagres de manzana, de vino blanco y de vino de Jerez. Del mismo modo, el análisis de componentes principales (PCA) permitió separar los diferentes tipos de vinagres según el perfil de aminas biógenas, a excepción de los vinagres de vino tinto.

Por otro lado, los cambios en el perfil de aminas biógenas fueron monitorizados durante el almacenamiento de botellas de vino abiertas. Para ello, se seleccionaron tres tipos de vino diferentes (vinos tintos de calidad media y de alta calidad y vino blanco joven). Las botellas de vino una vez abiertas se mantuvieron en diferentes condiciones de almacenamiento (temperatura y tipo de tapón). La concentración de aminas biógenas en los vinos recién abiertos variaron de

5.08 a 39.2 mg/L. En general, los vinos tintos presentaron mayores cantidades de histamina y putrescina, mientras que el vino blanco mostró una concentración similar para tiramina, putrescina e histamina. Por el contrario, la metilamina y la espermina no se detectaron en ninguna muestra. Aunque se observaron ligeros cambios en la concentración de algunas aminas biógenas en las diferentes condiciones de almacenamiento, ninguna de ellas aumentó de forma significativamente en ningún caso.

El análisis de componentes principales (PCA) permitió agrupar los diferentes tipos de vino usando las aminas biógenas como variables. Estos resultados fueron confirmados tras el análisis discriminante lineal (LDA), el cual pudo discriminar los tres tipos de vino según el tiempo de almacenamiento. Finalmente, se observó una marcada correlación positiva entre histamina, tiramina, putrescina y cadaverina considerando la concentración de aminas biógenas de todas las muestras ($r = 0.73$-0.94).

Artículo 4

A survey of biogenic amines in vinegars.

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Food Chemistry
141 (2013) 2713–2719

Q1; 6/130 (JCR-ISI, Food Science & Technology, 2016)
Impact Factor: 4.529

Food Chemistry 141 (2013) 2713–2719

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

A survey of biogenic amines in vinegars

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ARTICLE INFO

Article history:
Received 27 March 2012
Received in revised form 31 January 2013
Accepted 16 May 2013
Available online 31 May 2013

Keywords:
HPLC
AQC
Solid-phase extraction
Biogenic amines
Vinegar

ABSTRACT

This paper reports the determination of biogenic amines by high-performance liquid chromatography (HPLC) and fluorescence detection after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) in balsamic, apple, and red, white, and Sherry wine vinegars. A solid-phase extraction (SPE) with mixed-mode resins method was used before analysis. The method was successfully validated obtaining adequate values of selectivity, response linearity, precision, accuracy, and low detection and quantification limits. The total content of biogenic amines in vinegars ranged from 23.35 to 1445.2 μg/L, being lower than those reported in wines. Putrescine was the amine that showed the highest concentrations in most samples. Methylamine and phenylethylamine were not determined in any vinegar. Balsamic and "Pedro Ximénez" Sherry vinegars reached the highest amounts of biogenic amines, while apple, white and Sherry wine vinegars had the lowest concentrations. Principal component analysis using the biogenic amines as variables, allowed to separate the different kind of vinegars, excepting red vinegars.

1. Introduction

Amines are organic nitrogenous bases of low molecular weight, which are found especially in food and beverages from fermentative processes (Brink, Damink, Joosten, & Huis in't Veld, 1990; Kirschbaum, Rebscher, & Brückner, 1999). Most of these compounds are usually formed through the microbial decarboxylation of amino acids (Brink et al., 1990), which depend on the specific bacteria strain(s) present, the level of the carboxilase activity and the availability of the amino acids substrate (Rivas, González, Landete, & Muñoz, 2008; Suzzi & Gardini, 2003).

Despite the multiple physiological functions of biogenic amines, the consumption of food containing high amounts of these compounds can have toxicological effects and can cause problems in special consumers with a reaction to them (Brink et al., 1990; Silla, 1996). Hence, biogenic amines have been determined in a wide range of food products including fish, meat, vegetables, dairy products, nuts, chocolate, beer and wines (Brink et al., 1990; Lorenzo, Martinez, Franco, & Carballo, 2007).

Vinegar is one the most widespread and common product in the world because it is available in every country in several different varieties (Mazza & Murooka, 2009). It is derived from the conversion of ethanol to acetic acid by bacteria. It can therefore be produced from any alcoholic material, ranging from alcohol–water mixture to wine (Tesfaye, Morales, García-Parrilla, & Troncoso, 2002). Wine vinegar is the result of two fermentation processes, the conversion of sugars from must into ethanol by yeast and the oxidation of the ethanol by acetic acid bacteria (Callejón et al., 2009). Since biogenic amines are found especially in beverages from fermentative processes, it is interesting to know the levels of these compounds in vinegars. However, the determination of biogenic amines has been poorly studied in these products.

Analytical determination of biogenic amines is not simple due to the variety of their chemical structures and because they are usually present at low concentrations in complex matrices. High-performance liquid chromatography (HPLC) is the technique most often used to determine these amines due to its high resolution, sensitivity and simple preparation (Hernández-Orte, Peña-Gallego, Ibarz, Cacho, & Ferreira, 2006). However, biogenic amines do not have good absorption properties in the visible, ultraviolet or fluorescence wavelength ranges. For this reason, chemical derivatization prior to HPLC is used to the determination of these compounds (Peña-Gallego, Hernández-Orte, Cacho, & Ferreira, 2009). The derivatization reagents most widely used are: O-phthalaldehyde (OPA) (Hernández-Orte et al., 2006; Soleas, Carey, & Goldberg, 1999), dansyl chloride (Dns-Cl) (Hernández-Orte et al., 2006; Moret & Conte, 1996) or 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) (Busto, Guash, & Borrull, 1996; Hernández-Orte et al., 2006; Peña-Gallego et al., 2009), phenyl isothiocyanate (PITC) (Calull, Marcé, Fábregas, & Borrull, 1991), fluorenyl methyl chloroformate (FMOC) (Kouwatli, Chalom, Tod, Farinotti, & Mahuzier, 1992), among others. PITC derivatives cannot be detected by fluorescence and the methods used for them are less sensitive than those for other derivatization reagents. FMOC gives good sensitivity and stability derivatives, but it

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http://dx.doi.org/10.1016/j.foodchem.2013.05.087

produces multiples products from a single amine, so it is not suitable when dealing with complex samples or multiple standards. Dns-Cl is a non-specific reagent, since it can react with other substances such as phenols or aliphatic alcohols in addition to primary and secondary amine groups (Busto et al., 1996). On the other hand, OPA can react only with primary amines. It reacts easily within about 30 s in the presence of a reducing agent, and forms strong fluorescent derivatives increasing the selectivity of the method (Önal, 2007). However, OPA derivatives are not very stable, they no allow for clean-up after the derivatization processes and strict derivatization conditions are also necessary (Busto et al., 1996). AQC has shown to be a suitable derivatization agent for the determination of biogenic amines in wines (Busto et al., 1996; Hernández-Orte et al., 2006; Peña-Gallego et al., 2009). It reacts with primary and secondary amines giving stable fluorescent compounds and the excess of reagent is hydrolysed during the derivatization reaction avoiding interferences.

Most methods used in amine analysis are direct methods without previous treatments. However, the resulting chromatograms are complex because other compounds present in the samples can react with the derivatizing agent producing fluorescent compounds (Peña-Gallego et al., 2009). Hence, some methods using solid-phase extraction (SPE) prior derivatization of amines have been developed to avoid these interferences (Molins-Legua & Campins-Falcao, 2005). Recently, a SPE with mixed-mode resins method has been successfully developed for the determination of biogenic amines in wine (Peña-Gallego et al., 2009).

In this context, the aim of this study was the determination of biogenic amines in vinegar using SPE and AQC as derivatizing agent. For that, the method was validated and then was applied to different types of vinegars: balsamic, apple, and red, white, and Sherry wine vinegars.

2. Materials and methods

2.1. Reagents and standards

"AccQ-Fluor" Kit supplied by Waters (Milford, MA, USA) consists of 6-aminoquinolyl-N-hydrosysuccinimidyl carbamate reagent (AQC), acetonitrile to dissolve the reagent and 0.2 mM sodium borate buffer, pH 8.8. Biogenic amine standards were purchased from Sigma–Aldrich (Steinheim, Germany) and 2-aminoheptanoic acid (internal standard) was supplied by Fluka (Buchs, Switzerland). Calcium disodium EDTA and calcium chloride were supplied from Sigma–Aldrich (Steinheim, Germany). Sodium acetate and sodium hydroxide were obtained from Panreac (Castellar del Vallès, Barcelona) and trihydrate triethylamine (TEA), methanol (HPLC grade) and hydrochloric acid 32% (v/v) were acquired from Merck (Darmstadt, Germany). Orthophosphoric acid 85% (v/v) was purchased from Prolabo (Fontenay-sous-bois, France). Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

The amine standards were prepared individually dissolving the pure compounds in methanol (2 g/L), except agmatine and spermine which were dissolved in water. Concentrated solutions of amine standards (500 mg/L) were prepared by diluting the standard solution with water. The synthetic vinegar was an aqueous solution containing 7% (v/v) acetic acid.

2.2. Samples

A total of 26 vinegars were analysed: 6 red wine (RWV1-RWV6), 4 white wine (WWV1–WWV4), 10 sherry wine (VJ1–VJ3, VJR1–VJR3, VJGR1–VJGR3, PX), 3 balsamic (BV1–BV3), and 3 apple vinegars (AV1–AV3). RWV1 was the sample vinegar used for the validation of method. Among Sherry wine vinegar, one was a "Pedro Ximénez" (PX) Sherry vinegar. The rest of them belonged to the three categories established by the Sherry vinegar regulation in accordance with ageing time in oak barrels: "Vinagre Jerez" (VJ), minimum 6 month-old; "Reserva" (VJR) vinegars, at least 2 years-old; and "Gran Reserva" (VJGR) vinegars, at least 10 years-old (Dirección General de Industria y Mercados Alimentarios., 2009). Samples were obtained from markets (commercial vinegars), excepting two samples which were obtained directly from the wineries (RWV2 and VJGR1).

2.3. Solid-phase extraction (SPE) procedure

SPE procedure was performed following the method proposed by Peña-Gallego et al. (2009). Resin employed was Oasis MCX 1 cc (30 mg) Extraction Cartridges resins from Waters (MA, USA). Oasis MCX resins are composed of mixed-mode: reverse-phase (C18) and ion exchange sorbents (sulphonic groups). These were conditioned by passing 2 mL of methanol followed by 2 mL of Milli-Q water. Then, the sample (0.6 mL) was percolated. Three consecutive washings were made: (a) 2 mL of 10 mM H_3PO_4/MeOH (90:10) solution; (b) 2 mL of 10 mM NaOH/MeOH (70:30) solution; (c) 2 mL of 10 mM $CaCl_2$/MeOH (70:30) solution. Then, the analytes were eluted with 1.2 mL of the 100 mM NaOH/MeOH (65:35) solution. The eluate was collected in a vial containing 100 μL of 1.2 M HCl. Between each loading, washing and eluting stage, the column was washed with 1 mL of Milli-Q purified water.

2.4. Derivatization of standards and samples

Prior to the derivatization reaction, work solutions were prepared as follows:

In case of standards, 40 μL of internal standard (2-aminoheptanoic acid at a concentration of 2 mg/L) were added to 200 μL of the amine concentrated solutions and then the mixture was diluted adding 760 μL of Milli-Q purified water. In case of samples, they were not diluted due to their low levels of biogenic amines. Hence, they were prepared adding 40 μL of internal standard (2 mg/L) to 960 μL of sample after the extraction.

Then, the derivatization was performed by "AccQ-Fluor" kit according to the kit's instructions: 20 μL of work solution was buffered with 60 μL of a 0.2 M solution of sodium borate at pH 8.8. The derivatization reaction was made by adding 20 μL of the AQC solution.

2.5. Equipment

HPLC analysis was carried out in a Waters equipment consisting of an autosampler injector Waters 717, a Waters 600E system controller connected to a fluorescence detector, Waters 474. Data treatment was performed in a Waters Millennium data station. The column was a Luna C18, 5 μm, 250 × 4.6 mm and guard column 4.0 × 3.0 mm from Analytical Phenomenex, (Torrance, CA, USA).

Detection was carried out by fluorescence with excitation at 250 nm and emission at 395 nm. The injection volume was 10 μL and the separation was obtained at a flow rate of 1 mL/min at 65 °C with a gradient program employed by Hernández-Orte et al. (2006).

Mobile phase A consisted of a 140 mM solution of sodium acetate trihydrate and 17 mM of TEA adjusted to pH 5.05 and mobile phase B was methanol. All mobile phase were filtered through a membrane filter with a mean pore size of 0.45 μm (Millipore) prior to use.

2.6. Statistical analysis

All statistical analyses were performed by means of Statistica software (StatSoft, 2001). One-way ANOVA was performed to evaluate significant differences among types of vinegars (significance levels $p < 0.05$). Principal Component Analysis (PCA) was carried out as unsupervised method to ascertain the degree of differentiation between samples and the compounds were accounting for the differences.

3. Results and discussion

3.1. Method validation

The analytical method developed by Peña-Gallego et al. (2009) was adapted and validated for the determination of biogenic amines in vinegar. The validation parameters studied were the selectivity, response linearity, sensitivity (detection and quantification limits), precision (repeatability and intermediate precision) and accuracy (recovery studies).

The selectivity was tested using a vinegar sample. The selectivity criterion was that the resolution between adjacent peaks (R) was at least 1.5 (González & Herrador, 2007). In our case, R values obtained for all the peaks were higher than 1.5. Therefore, acceptable peak resolutions for biogenic amines were achieved (Fig. 1).

The linearity of the method was determined by regression analysis of the relative area (ratio between peak area of biogenic amines to the peak area of the internal standard) versus the amine concentration. For that, eight standards solutions of amines in concentrations ranging from 20 to 1000 μg/L were prepared and analysed in triplicate. Results of regression analysis and correlation analysis (R^2) are shown in Table 1. For all the biogenic amines, R^2 were above 0.991, showing a linear relation between the standards concentration and the detector response.

According to Peña-Gallego et al. (2009), the limits of detection (LDD) and quantitation (LDQ) were calculated based on the ratio $3S_{bo}/m$ and $10S_{bo}/m$, respectively. S_{bo} is the uncertainty of the background noise, which is calculated from the absolute area of the background noise measured around each amine divided by the area of the internal standard, and m is the slope of the respective linear calibration. As shown in Table 1, the limits of detection ranged from 7 to 26 μg/L and the limits of quantification were between 17 and 68 μg/L. Comparing to the literature, LDD of most amines were of the same order than those reported by other authors who also used AQC (Busto et al., 1996; Hernández-Orte et al., 2006) and lower than those obtained by other methods (Gómez-Alonso, Hermosín-Gutiérrez, & García-Romero, 2007; Herbert, Santos, & Alves, 2001). Specifically, LDD and LDQ for histamine, tyramine putrescine and cadaverine were similar or even lower than those obtained by Peña-Gallego et al. (2009). Hence, the analytical method showed to be sensitive enough for the determination of biogenic amines.

The precision (repeatability and intermediate precision) was expressed as relative standard deviation (RSD) and were calculated employing a vinegar sample diluted with water (65% v/v) and spiked with 1 mg/L of each standard.

The repeatability of the method was studied injecting five successive of a derivatized spiked vinegar and the relative standard deviation ranged from 0.5 for methylamine to 6.7 for spermine (Table 1). These values were lower than those obtained by Peña-Gallego et al. (2009) except for putrescine, which showed a RSD of 6.6. The study of intermediate precision was performed along a period of 7 days in which the derivatized spiked vinegar was injected in duplicates at five working sessions. The values obtained ranged from 2.7 for spermine to 10.9 for cadaverine (Table 1). Results of repeatability and intermediate precision are in agreement with the values proposed by AOAC (AOAC, 1993).

Recovery was determined by adding two quantities (0.5 and 5 mg/L) of each amine to a vinegar sample diluted with water (65%). Results of this study can be seen in Table 1. A good degree of accuracy was achieved for most of the compounds, reaching recovery percentages up to 80% and lower than 110%. These are within the acceptable range for AOAC for concentration between 0.1 and 10 mg/L (AOAC, 1993). Only putrecine, agmatine at low addition level and cadaverine at high level showed recovery values below 80% (73.6%, 65.3%, and 67.4%, respectively).

3.2. Sample analysis

The proposed method was applied for the determination of biogenic amines in different types of vinegars. The analysis

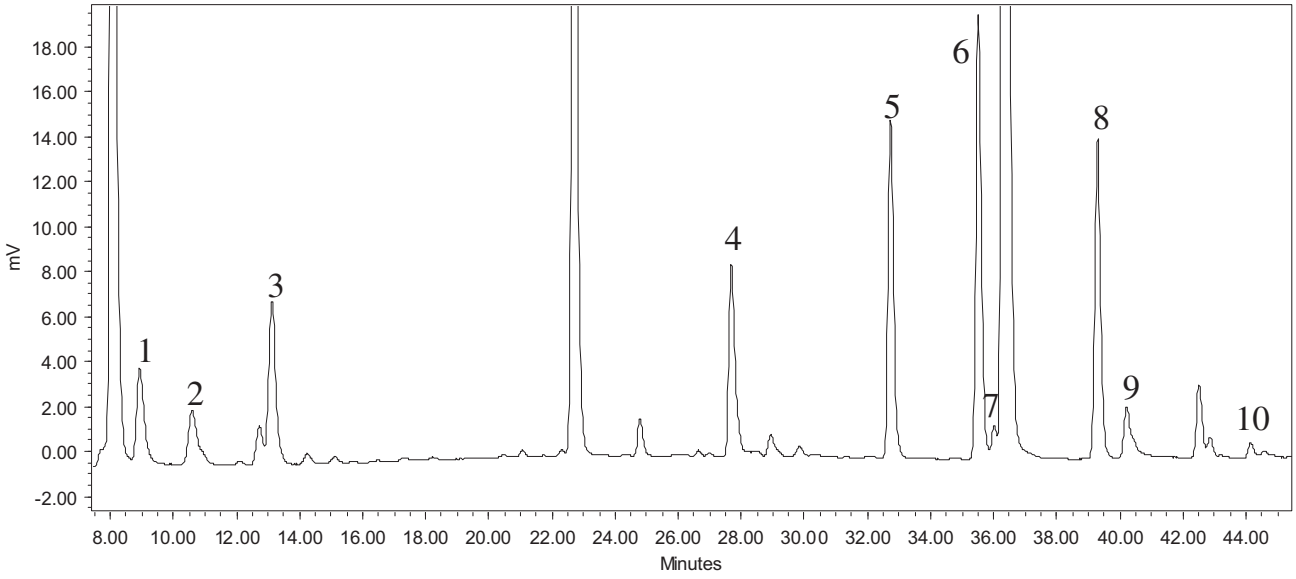

Fig. 1. Chromatogram of a standard solution. (1) Histamine, (2) agmatine, (3) methylamine, (4) tyramine, (5) putrescine, (6) cadaverine, (7) internal standard, (8) phenylethylamine, (9) spermidine, (10) spermine.

Table 1
Validation of the method.

Compound	RT (min)	Linear range (μg/L)	Slope	Intercept	R^2	LOD (μg/L)	LOQ (μg/L)	Repeatability (n = 5)		Intermediate precision (n = 5)		Low: vinegar + 0.5 mg/L		High: vinegar + 5 mg/L	
								Mean (mg/L)	RSD (%)	Mean (mg/L)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Histamine	8.943	100–750	0.079 ± 0.001	+2.7741	0.999	15	51	1.3	2.1	1.7	7.7	88.8	6.3	95.0	3.2
Agmatine	10.613	100–750	0.059 ± 0.003	−1.2284	0.993	20	68	0.7	2.5	0.7	5.1	65.3	6.2	99.5	3.7
Methylamine	13.130	100–750	0.161 ± 0.005	+1.7355	0.996	19	64	1.4	0.5	1.9	5.0	96.3	9.5	98.2	7.0
Tyramine	27.682	100–750	0.119 ± 0.004	+3.5711	0.997	26	57	1.6	2.0	1.8	8.7	80.3	6.4	95.0	7.8
Putrescine	32.721	50–750	0.267 ± 0.008	+1.7481	0.997	12	39	0.9	6.6	0.7	10.5	73.6	2.2	99.8	2.4
Cadaverine	35.991	20–250	0.624 ± 0.020	−13.7996	0.997	7	17	0.7	1.0	0.8	10.9	80.1	9.4	67.4	12.7
Phenylethylamine	39.278	100–750	0.267 ± 0.005	+6.2198	0.999	12	39	1.3	1.6	1.5	8.7	106.2	2.1	86.7	9.0
Spermidine	40.205	100–750	0.160 ± 0.004	+5.4470	0.997	19	64	0.9	1.3	1.0	8.1	87.9	10.3	96.7	9.5
Spermine	44.788	100–1000	0.085 ± 0.003	−9.3447	0.991	14	48	1.4	6.7	1.4	2.7	89.2	12.9	98.6	3.3

RT, retention time; R^2, correlation coefficients; LOD, limits of detection; LOQ, limits of quantification; RSD, relative standard deviation.

results of these samples are summarised in Table 2. The total content of biogenic amines in vinegars ranged from 23.35 to 1445.2 μg/L (Table 2). These amounts were lower than found in wines, since total amounts of amines in these products can reach even 130 mg/L (Soufleros, Barrios, & Bertrand, 1998). There is little information on the biogenic amine production by acetic bacteria in the literature. In fact, we are only aware of one work in which no strains of acetic bacteria were able to produce biogenic amines in synthetic medium, wine and grape must (Landete, Ferrer, & Pardo, 2007). On the other hand, according to Ancín-Azpilicueta, González-Marco, and Jiménez-Moreno (2008), some amines, such as histamine and tyramine, can be degraded by oxidase enzymes present in some bacteria. Hence, these two facts could explain the lower amounts of biogenic amines found in vinegars.

In general, methylamine and phenylethylamine were not determined in any vinegar. According to some authors, the concentration of volatile amines seems to decrease during fermentation process (Ancín-Azpilicueta et al., 2008; Herbert, Cabrita, Ratola, Laureano, & Alves, 2005) probably due to their volatility or their metabolization during the fermentation (Herbert et al., 2005). These facts could also explain the no presence of methylamine and phenylethylamine amines in vinegars. Although cadaverine was present in a higher number of samples, putrescine followed by histamine were the amines that showed the highest concentrations, reaching values up to 525 and 309 μg/L, respectively (Table 2). These results were expected since these amines were the most abundant in wines (Herbert et al., 2005; Hernández-Orte et al., 2006; Peña-Gallego et al., 2009). High levels of putrescine and histamine in wine may reflect poor hygiene conditions during winemaking (Konakovsky et al., 2011). Nevertheless, these levels may be of difficult definition because of the wide range of concentrations found in wines (Soufleros et al.,

Table 2
Concentration of biogenic amines in vinegars.

Type of vinegar	Samples	Mean concentration (μg/L) ± Standard deviation									
		Histamine	Agmatine	Methylamine	Tyramine	Putrescine	Cadaverine	Phenylethylamine	Spermidine	Spermine	Total
Red wine	RWV1	n.q.	n.q.	n.d.	n.d.	79 ± 3	n.d.	n.d.	n.d.	n.d.	79
	RWV2	n.q.	n.q.	n.d.	n.d.	51.9 ± 0.4	27.12 ± 0.03	n.d.	n.d.	137.7 ± 0.2	216.72
	RWV3	150 ± 3	n.q.	n.d.	n.q.	166 ± 2	51.7 ± 0.2	n.d.	154 ± 3	116.97 ± 0.04	638.67
	RWV4	129.1 ± 1.2	n.q.	n.d.	n.q.	208.9 ± 1.4	60.4 ± 0.3	n.d.	n.d.	115.1 ± 0.2	513.5
	RWV5	n.q.	n.q.	n.d.	n.d.	78.2 ± 0.4	n.d.	n.d.	n.q.	n.d.	78.2
	RWV6	258 ± 6	100 ± 10	n.d.	n.q.	276 ± 6	25.00 ± 0.14	n.d.	101 ± 2	n.d.	760
White wine	WWV1	n.q.	n.q.	n.d.	n.d.	54 ± 2	25.0 ± 0.1	n.d.	n.d.	115.2 ± 0.2	194.2
	WWV2	n.d.	n.q.	n.d.	n.d.	57.0 ± 0.4	23.96 ± 0.03	n.d.	n.d.	119.29 ± 0.13	200.25
	WWV3	n.q.	n.q.	n.d.	n.d.	n.q.	27.4 ± 0.3	n.d.	n.q.	129.0 ± 0.1	156.4
	WWV4	n.d.	n.q.	n.d.	n.d.	104 ± 2	n.d.	n.d.	n.d.	119.26 ± 0.05	223.26
Sherry wine	VJ1	n.d.	n.q.	n.d.	n.d.	77.4 ± 0.6	23.76 ± 0.05	n.d.	n.q.	n.d.	101.16
	VJ2	n.d.	n.q.	n.d.	n.d.	120 ± 10	28.4 ± 0.1	n.d.	n.d.	n.d.	148.4
	VJ3	n.d.	n.q.	n.d.	n.d.	120 ± 10	24.20 ± 0.04	n.d.	n.d.	n.d.	144.2
"Reserva" sherry wine	VJR1	n.d.	n.q.	n.d.	n.d.	61.8 ± 0.5	23.3 ± 0.1	n.d.	n.d.	n.d.	85.1
	VJR2	n.d.	n.q.	n.d.	n.d.	148.3 ± 2.3	23.41 ± 0.01	n.d.	n.d.	n.d.	171.71
	VJR3	n.d.	n.q.	n.d.	n.d.	n.d.	23.43 ± 0.03	n.d.	n.d.	n.d.	23.43
"Gran Reserva" sherry wine	VJGR1	153.1 ± 1	n.q.	n.d.	n.q.	161.1 ± 0.8	23.40 ± 0.03	n.d.	n.d.	n.d.	337.6
	VJGR2	n.d.	nq	n.d.	n.d.	75 ± 1	23.11 ± 0.01	n.d.	n.d.	n.d.	98.11
	VJGR3	n.q.	n.q.	n.d.	n.d.	n.q.	24.52 ± 0.03	n.d.	n.q.	n.d.	24.52
Pedro Ximénez	PX	253 ± 26	166.3 ± 0.1	n.q.	n.q.	115.9 ± 0.1	27.68 ± 0.04	n.d.	131 ± 3	n.d.	693.88
Balsamic	BV1	163 ± 3	125.5 ± 2.3	n.d.	n.q.	51.5 ± 0.5	27.53 ± 0.01	n.d.	124 ± 3	n.d.	491.53
	BV2	190.2 ± 0.6	210 ± 30	n.d.	n.d.	205.0 ± 1.5	23.52 ± 0.02	n.d.	157.5 ± 2.5	n.d.	786.22
	BV3	309 ± 6	147.0 ± 1.1	n.d.	216.2 ± 0.4	525 ± 6	24.7 ± 0.2	n.d.	223.3 ± 2.2	n.d.	1445.2
Apple	AV1	n.d.	n.q.	n.d.	n.d.	n.d.	23.35 ± 0.02	n.d.	n.d.	n.d.	23.35
	AV2	n.q.	n.q.	n.d.	n.d.	n.q.	24.11 ± 0.01	n.d.	n.d.	n.d.	24.11
	AV3	n.q.	n.q.	n.d.	n.d.	n.d.	24.1 ± 0.1	n.d.	n.d.	n.d.	24.1

n.q., non quantified; n.d., non detected.

1998). In general, 8–40 mg of histamine can cause slight poisoning, over 40 mg moderate, while over 100 mg severe poisoning (Parente et al., 2001). Putrescine has no adverse health effect but can increase the potential toxicity of histamine (Hernández-Orte et al., 2006). The concentration of these amines in vinegars was much lower than these values. Hence, the potential toxicity of these amines in vinegar is practically negligible.

Red wine vinegars were the samples which showed more differences among them. The total content of biogenic amines ranged from 78.2 to 760 μg/L (Table 2). The last value corresponds to the RWV6 sample which stood out for its higher concentrations of histamine and putrescine. This variability in the concentration of amines in red wine vinegars is due to numerous factors such as the variety of grape, the method of winemaking employed, the specific bacterial strain present and the availability of free amino acids (Ancín-Azpilicueta et al., 2008; Herbert et al., 2005; Naila, Steve, Graham, & Meerdink, 2010). In fact, some authors found that wines produced from the varieties with the highest concentrations of assimilable amino acids were those with the highest concentration of biogenic amines (Herbert et al., 2005).

The total content of biogenic amines in white wine vinegars was more similar and lower than values found for most red wine vinegars, ranging from 156.4 to 223.26 μg/L. These results are in agreement to several authors, since amine levels in red wines are usually higher than in white wines due to the different winemaking method employed (Ancín-Azpilicueta et al., 2008). In fact, high pH and malolactic fermentation have an important influence in the generation of amines. Thus, red wines are generally less acidic and furthermore, in wine-making, malolactic fermentation usually has a greater importance than in white wines (Romero, Sánchez-Viñas, Gázquez, & Bagur, 2002). As shown in Table 2, in addition to methylamine and phenylethylamine, histamine, agmatine, tyramine and spermidine were not quantified in any white wine vinegar. Several authors suggested that histamine and tyramine are formed primarily during the malolactic fermentation (Konakovsky et al., 2011; Soufleros et al., 1998), and this could explain the not quantified levels of these amines in white wine vinegars. On the contrary, spermine was quantified in all white wine vinegars and cadaverine and putrescine in most of them. These amines presented similar concentrations in almost all white wine vinegar samples. According to Soufleros et al. (1998), cadaverine seems that is not very influenced by malolactic fermentation and this could be a possible reason why this amine is present in most of the white wine vinegars.

Sherry vinegars also showed low concentrations of total biogenic amines, ranging from 23.43 to 337.6 μg/L (Table 2). These values seem be similar to those found in white wine vinegar, and therefore, lower than values of most red wine vinegars. This result was expected since, Sherry wines showed to have low biogenic amines levels compared with red wines (Moreno-Arribas & Polo, 2008). In these samples, agmatine, tyramine, spermidine and spermine were not quantified in any Sherry vinegar. On the contrary, cadaverine was the only amine quantified in all samples and putrescine in most of them (Table 2). This is in agreement to Moreno-Arribas and Polo (2008), since the highest mean values were recorded for putrescine and cadaverine during the biological ageing of Sherry wines. Comparing the three different ageing categories of Sherry wine vinegars, we could say that the time of ageing in wood barrel had not a clear influence on the biogenic amines profile. Cadaverine presented a fairly constant concentration in all the samples in agreement to values found in Sherry wines (Moreno-Arribas & Polo, 2008). Excepting VJGR1, histamine values were below detection limits.

Pedro Ximénez Sherry vinegar (PX) showed significant differences in total values of biogenic amines, being higher than those reached by the rest of Sherry vinegars (Table 2). This fact could be due to the addition of Pedro Ximénez wine during the elaboration process of PX vinegar (Dirección General de Industria y Mercados Alimentarios, 2009). Furthermore, this addition of Pedro Ximénez wine can also explain that Sherry vinegar reached significant higher amounts of total biogenic amines than all white wines and most of red wine vinegars (Table 2). Among the amines determined, histamine was the mayor amine in the PX vinegar, reaching even significantly higher concentrations than all analysed vinegars, excepting the balsamic ones. Spermidine could be quantified in PX vinegar, unlike Sherry vinegars. On the other hand, spermine and tyramine were not quantified in this PX sample (Table 2).

In general, apple vinegars showed the lowest amounts of total biogenic amines, being significant with respect to white wine, PX and balsamic vinegars (Table 2). This result was expected since the total mean biogenic amines levels found in ciders were lower than values reported in wines (Garai, Dueñas, Irastorza, Martín-Álvarez, & Moreno-Arribas, 2006). Most of amines were present under quantification and detection limits. Only cadaverine could be quantified in all apple vinegar samples, but reaching very low concentrations (Table 2).

On the contrary, balsamic vinegars, along with the PX, were the samples with the highest contents in biogenic amines. Balsamic vinegars are produced from the concentrated must of *Trebbiano* or other local grapes. There are two kinds of balsamic vinegars, "aceto balsamico tradizionale di Modena" and "aceto balsamico di Modena". The samples analysed in this study belong to the last type ("aceto balsamico di Modena"), which are produced by a double fermentation with a possible addition of wine vinegar during its manufacture (Erbe & Brückner, 1998). The concentration of total biogenic amines in these vinegars ranged from 491.53 to 1445.2 μg/L, being significantly higher than those found in apple, Sherry and white wine vinegars (Table 2). This could due to the total amino acid content since, according to Erbe and Brückner (1998), balsamic vinegars showed much higher amounts of amino acids than Sherry and cider.

Among the biogenic amines analysed, spermine was not detected in any balsamic samples. On the contrary, histamine was the amine most abundant, showing similar concentration to PX and significantly higher than all apple, Sherry and white wine vinegars (Table 2). Putrescine also reached high concentration, but no similar concentration was observed, ranging from 50 to 500 μg/L. Furthermore, it must be emphasised the high amounts of agmatine and spermidine in balsamic vinegars. In fact, these two amines reached significant differences in their concentrations with respect to all vinegar except PX.

3.3. Principal component analysis

Principal component analysis (PCA) was performed to evaluate whether the profile of biogenic amines were great enough to distinguish the different kind of vinegars analysed in this study. First, we carried out the PCA considering all samples. However, we observed the WWV4 sample was not placed near the other white wine vinegars due to this sample did not contain cadaverine and, was subsequently distorting the analysis. Hence, we decided to repeat the PCA removing this sample, obtaining that the principal four components explained 94% of the cumulative variance. The corresponding scores and loadings are plotted into the plan made up of the first two principal components in Fig. 2. PC1 seems to separate the samples according to total amounts of biogenic amines. Thus, apple, Sherry and white wine vinegars were placed on the right side of the graph, while those vinegars richer in these amines (balsamic and PX vinegar) were on the left. As mentioned above, red wine vinegars showed a wide range of concentration. For that reason, all of them are not placed in the same quadrant. On the other hand, PC2 seems to separate the samples according

to the presence or absence of spermine. Samples with spermine, such as white and some red wine vinegars, were separated from the rest of them. As consequence of PCA results, the samples appear distributed in different separated groups. Apple and most Sherry vinegars were together, since they presented lower concentrations of biogenic amines and did not show spermine. Only VJGR1 was placed along to the group formed by balsamic and PX vinegars, since it was the Sherry vinegar with the highest concentrations in biogenic amines and it was the only one that presented histamine. This could be possible due this vinegar was obtained from the winery, and their ageing time was much higher than the rest of Sherry vinegars. White wine vinegars were related to spermine and, for that, they were separated from apple and Sherry vinegars despite their low concentrations of biogenic amines.

As mentioned above, red wine vinegars were the only samples that do not appear together due to their different amounts and profile of biogenic amines. RWV2, RWV3 and RWV4 were related to cadaverine and spermine. However, the first one appears in other quadrant, together the white wine vinegars, since histamine was not quantified. Spermine and cadaverine were not quantified in

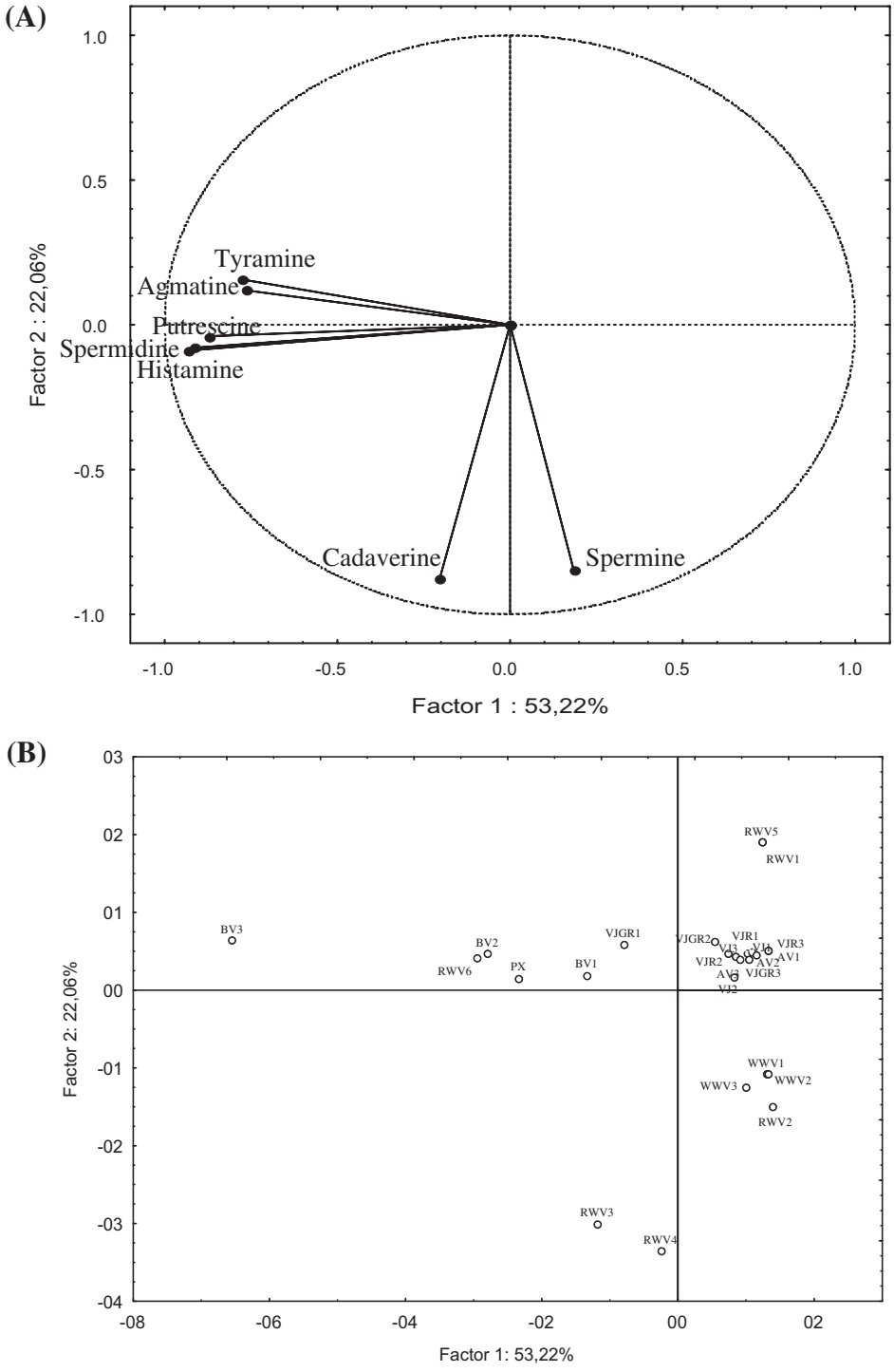

Fig. 2. Graphic of scores (A) and loadings (B) into the plan made up of the first two principal components.

RWV1 and RWV5, and for that reason were placed in the contrary quadrant of RWV2. Finally, RWV6 was the only red wine vinegar with agmatine. Hence, it was the only red wine vinegar placed next to the balsamic ones.

4. Conclusions

A method for the determination of biogenic amines in vinegar was successfully validated obtaining adequate values to selectivity, response linearity, precision and accuracy, as well as low detection and quantification limits. Its utility for the routine analysis of biogenic amines in vinegar has been proved. In general, the concentration of biogenic amines in vinegars was lower than found in wines. These results suggest that biogenic amines are not produced and even degraded during acetic fermentation. Hence, further studies about the evolution of biogenic amines during the elaboration of vinegar are necessary to confirm this hypothesis. Among analysed vinegars, balsamic and "Pedro Ximénez" Sherry vinegars reached the highest amounts of biogenic amines, while apple vinegar had the lowest concentrations. These results were confirmed by the analysis of PCA, since the different kinds of vinegars were separated using the biogenic amines as variables, excepting red wine vinegars due to their different amines profile.

Acknowledgements

We wish to thanks the Ministry of Science and Innovation of the Spanish government for the financial support of project AGL2010-22152-C03-01 and the Junta de Andalucía for the project AGR P07-02480.

References

Ancín-Azpilicueta, C., González-Marco, A., & Jiménez-Moreno, N. (2008). Current knowledge about the presence of amines in wine. *Critical Reviews in Food Science and Nutrition, 48*, 257–275.

AOAC (1993). *Peer verified methods program. Manual on policies and procedures*.

Brink, B. T., Damink, C., Joosten, H., & Huis in't Veld, J. (1990). Occurrence and formation of biologically active amines in foods. *International Journal of Food Microbiology, 11*, 73–84.

Busto, O., Guash, J., & Borrull, F. (1996). Determination of biogenic amines in wine after precolumn derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. *Journal of Chromatography A, 737*, 205–213.

Callejón, R. M., Tesfaye, W., Torija, M. J., Mas, A., Troncoso, A. M., & Morales, M. L. (2009). Volatile compounds in red wine vinegars obtained by submerged and surface acetification in different woods. *Food Chemistry, 113*, 1252–1259.

Calull, M., Marcé, R. M., Fábregas, J., & Borrull, F. (1991). A method for the determination of histamine in wine by hplc with precolumn derivatization with phenylisothiocyanate. *Chromatographia, 31*, 133–136.

Dirección General de Industria y Mercados Alimentarios. (2009). Resolución de 24 de Marzo de 2009 por la que se concede la protección nacional transitoria a la Denominación de Origen Protegida "Vinagre de Jerez". *Boletín Oficial del Estado de 13 de Abril de 2009, Núm. 90*, 34603–34613.

Erbe, T., & Brückner, H. (1998). Chiral amino acid analysis of vinegars using gas chromatography–selected ion monitoring mass spectrometry. *Food Research and Technology, 207*, 400–409.

Garai, G., Dueñas, M. T., Irastorza, A., Martín-Álvarez, P. J., & Moreno-Arribas, M. V. (2006). Biogenic amines in natural ciders. *Journal of Food Protection, 69*, 3006–3012.

Gómez-Alonso, S., Hermosín-Gutiérrez, I., & García-Romero, E. (2007). Simultaneous HPLC analysis of biogenic amines, amino acids, and ammonium ion as aminoenone derivatives in wine and beer samples. *Journal of Agricultural and Food Chemistry, 55*, 608–613.

González, A. G., & Herrador, M. A. (2007). A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles. *Trends in Analytical Chemistry, 26*, 227–238.

Herbert, P., Cabrita, M. J., Ratola, N., Laureano, O., & Alves, A. (2005). Free amino acids and biogenic amines in wines and musts from the Alentejo region. Evolution of amines during alcoholic fermentation and relationship with variety, sub-region and vintage. *Journal of Food Engineering, 66*, 315–322.

Herbert, P., Santos, L., & Alves, A. (2001). Simultaneous quantification of primary, secondary amino acids and biogenic amines in musts and wines using OPA/3-MPA/FMOC-Cl fluorescent derivatives. *Journal of Food Science, 66*, 1319–1325.

Hernández-Orte, P., Peña-Gallego, A., Ibarz, M. J., Cacho, J., & Ferreira, V. (2006). Determination of the biogenic amines in musts and wines before and after malolactic fermentation using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate as the derivatizing agent. *Journal of Chromatography A, 1129*, 160–164.

Kirschbaum, J., Rebscher, K., & Brückner, H. (1999). Determination of biogenic amines in fermented beverages and vinegars by pre-column derivatization with para-nitrobenzyloxycarbonyl chloride (PNZ-CI) and reversed-phase LC. *Chromatographia, 49*, 117–124.

Konakovsky, V., Focke, M., Hoffmann-Sommergruber, K., Schmid, R., Scheiner, O., Moser, P., et al. (2011). Levels of histamine and other biogenic amines in high-quality red wines. *Food Additives and Contaminants, 28*, 408–416.

Kouwatli, H., Chalom, J., Tod, M., Farinotti, R., & Mahuzier, G. (1992). Precolumn derivatization of amines in liquid chromatography using luminescent probes: Comparison of several reagents with luminarin 1. *Analytica Chimica Acta, 266*, 243–249.

Landete, J. M., Ferrer, S., & Pardo, I. (2007). Biogenic amine production by lactic acid bacteria, acetic bacteria and yeast isolated from wine. *Food Control, 18*, 1569–1574.

Lorenzo, J. M., Martinez, S., Franco, I., & Carballo, J. (2007). Biogenic amine content during the manufacture of dry-cured lacón, a Spanish traditional meat product: Effect of some additives. *Meat Science, 77*, 287–293.

Mazza, S., & Murooka, Y. (2009). Vinegar through the age. In L. Solieri & P. Giudici (Eds.), *Vinegars of the world* (pp. 17–39). Millan: Spinger-Verlag.

Molins-Legua, C., & Campins-Falcao, P. (2005). Solid phase extraction of amines. *Analytica Chimica Acta, 546*, 206–220.

Moreno-Arribas, M. V., & Polo, M. C. (2008). Occurrence of lactic acid bacteria and biogenic amines in biologically aged wines. *Food Microbiology, 25*, 875–881.

Moret, S., & Conte, L. S. (1996). High-performance liquid chromatographic evaluation of biogenic amines in foods—An analysis of different methods of sample preparation in relation to food characteristics. *Journal of Chromatography A, 729*, 363–369.

Naila, A., Steve, F., Graham, F., & Meerdink, G. (2010). Control of biogenic amines in food-existing and emerging approaches. *Journal of Food Science, 75*, 139–150.

Önal, A. (2007). Current analytical methods for the determination of biogenic amines in foods. *Food Chemistry, 103*, 1475–1486.

Parente, E., Matuscelli, M., Gadrini, F., Grieco, S., Crudele, M. A., & Suzzi, G. (2001). Evolution of microbial populations and biogenic amines production in dry sausages produced in southern Italy. *Journal of Applied Microbiology, 90*, 882–891.

Peña-Gallego, A., Hernández-Orte, P., Cacho, J., & Ferreira, V. (2009). Biogenic amine determination in wines using solid-phase extraction: A comparative study. *Journal of Chromatography A, 1216*, 3398–3401.

Rivas, R., González, R., Landete, J. M., & Muñoz, R. (2008). Characterization of a second ornithine decarboxylase isolated from *Morganella morganii*. *Journal of Food Protection, 71*, 657–661.

Romero, R., Sánchez-Viñas, M., Gázquez, D., & Bagur, M. G. (2002). Characterization of selected Spanish table wine samples according to their biogenic amine content from liquid chromatographic determination. *Journal of Agricultural and Food Chemistry, 50*, 4713–4717.

Silla, M. H. (1996). Biogenic amines: Their importance in foods. *International Journal of Food Microbiology, 29*, 213–231.

Soleas, G. J., Carey, M., & Goldberg, D. M. (1999). Method development and cultivar-related differences of nine biogenic amines in Ontario wines. *Food Chemistry, 64*, 49–58.

Soufleros, E., Barrios, M. L., & Bertrand, A. (1998). Correlation between the content of biogenic amines and other wine compounds. *American Journal Enology and Viticulture, 49*, 266–278.

Suzzi, G., & Gardini, F. (2003). Biogenic amines in dry fermented sausages: A review. *International Journal of Food Microbiology, 88*, 41–54.

Tesfaye, W., Morales, M. L., García-Parrilla, M. C., & Troncoso, A. M. (2002). Evolution of phenolic compounds during an experimental aging in wood of sherry vinegar. *Journal of Agricultural and Food Chemistry, 50*, 7053–7061.

Artículo 5

Evaluation of biogenic amines profile in wine bottles: impact of storage conditions.

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Journal of Food Composition and Analysis
63 (2017) 139-147

Q1; 24/130 (JCR-ISI, Food Science & Technology, 2016)
Impact Factor: 2.752

Journal of Food Composition and Analysis 63 (2017) 139–147

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Original research article

Evaluation of biogenic amines profile in opened wine bottles: Effect of storage conditions

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ARTICLE INFO

Keywords:
Wines
Biogenic amines
HPLC
Nitrogen compounds
Solid phase extraction
Wine-triggered headache
AQC
Storage conditions
Food analysis
Food composition

ABSTRACT

Changes in biogenic amines profile during storage of opened wine bottles have been monitored. Three different wine types were selected (standard and high quality red wines and young white wine), and once opened, wine bottles were kept in different storage conditions (temperature and stopper kind). The total concentration of biogenic amines in just opened bottles ranged between 35.4–39.2 mg/L in standard quality red wine; 18.5–22.8 mg/L in high quality red wine; and 5.08 mg/L in bulk young white wine. Both red wines showed higher amounts of histamine and putrescine, whilst the white wine contained tyramine, putrescine and histamine in a similar concentration. Cadaverine presented a similar concentration for all samples (0.25–0.42 mg/L). Methylamine and spermine were not detected in any sample. The results from 36 samples showed that biogenic amines did not increase significantly in any case. Slight changes in the concentration of certain biogenic amines were detected as a result of the different storage conditions. A principal component analysis using biogenic amines as variables allowed to group the different kinds of wine. Linear Discriminant Analyses grouped the three wine types by storage time. Histamine and cadaverine showed a marked positive correlation in all wine types.

1. Introduction

Biogenic amines are generally considered as a food hazard, even though there is not a threshold for these biomolecules in the European legislation, except for histamine in fishery products (European Commission Regulation (EC) 2073/2005). Recently, a scientific opinion elaborated by the European Food Safety Authority (EFSA) remarked the risk associated with the synthesis of biogenic amines in fermented products, such as cheese, wine, beer, fermented sausages, among others (EFSA, 2011). Thus, histamine is the most widely studied amine due to its ability to produce headaches, hypotension and digestive problems, while tyramine is often associated with migraine and hypertension (Maintz and Novak, 2007). The relation between these biogenic amines in wine and headache remains controversial (Krymchantowski and Da Cunha Jevoux, 2014; Panconesi, 2008), although they may effect a synergistic action (Maintz and Novak, 2007). Furthermore, the effects of these amines may be potentiated by other biogenic amines such as putrescine, cadaverine and agmatine (EFSA, 2011).

Biogenic amines are organic nitrogenous bases of low molecular weight that have been detected and quantified in a great deal of fermented foods and are mainly formed by microbial decarboxylation of some amino acids. On the other hand, volatile amines can be formed by amination and transamination of aldehydes and ketones (Peña-Gallego et al., 2012). Biogenic amines are stable compounds and once they are formed it is difficult to eliminate them (EFSA, 2011).

For all the reasons mentioned above, literature on biogenic amines in different food products, especially in fermented products, is extensive. The concentration of biogenic amines in fermented products depends mainly on three factors: specific bacteria strain(s) present in the fermentation media, carboxylase activity and the amount of precursor amino acids in the substrate (De Las Rivas et al., 2008).

Besides, other factors may play an important role in the final concentration of biogenic amines in wine. Thus, nitrogenous fertilization, climatic conditions during growth, grape variety, geographic location or the level of maturation may cause changes in the amino acids profile in grapes. Moreover, the amino acids concentration may be altered by different prefermentative treatments such as clarification, crushing or duration of maceration process (Herbert et al., 2005). On the other hand, some factors involved in alcoholic and malolactic fermentations, such as pH, temperature, SO_2 concentration, turbidity or volatile acidity may also affect the concentration of biogenic amines (Herbert et al., 2005).

Wine has specifically been studied throughout its different stages of elaboration and storage. Thus, the concentration of biogenic amines has been determined in grapes (Bauza et al., 2007); musts (Del Prete et al., 2009; García-Marino et al., 2010; Rodriguez-Naranjo et al., 2013; Wang

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http://dx.doi.org/10.1016/j.jfca.2017.07.042
Received 21 December 2015; Received in revised form 5 May 2016; Accepted 29 July 2017
Available online 31 July 2017

Table 1
Derivatization reagent, type of detection, LOD and concentrations of biogenic amines in grape musts, wines and wine vinegars from different countries.

Der reagent	Detection	His (mg/L)	Tyr (mg/L)	Put (mg/L)	Cad (mg/L)	Agm (mg/L)	Met (mg/L)	Phe (mg/L)	Spm (mg/L)	Spmd (mg/L)	Total biogenic amines (mg/L)	LOD (mg/L)	Type and country	References
OPA	Fl $\lambda_{ex/em}$: 340/426 nm	1.7–14.9	n.d. – 6.7	0.5–25.0	n.q. – 2.1	n.q.	–	n.q. – 1.35	–	–	7.5–46.6	0.015–0.11	Red wine Spain	García-Marino et al. (2010)
	Fl $\lambda_{ex/em}$: 340/425 nm	n.d. – 18.7	1.1–17.8	7.6–35.7	n.d.	–	n.d.	–	–	–	25.2–96.8	0.05–0.35	Red wine Spain	Arrieta and Prats-Moya (2012)
	Fl $\lambda_{ex/em}$: 340/420 nm	0.3–2.5	0.1 – 0.8	2.3–4.0	0.3 – 0.5	–	–	1.0–2.1	–	–	6.0–12.0	–	Red wine Spain	López et al. (2012)
DNS-CL	UV-vis λ: 220 nm	0.5–27.0	1.1- 10.7	2.93–122	n.d. – 3.27	–	–	n.d. – 1.7	–	0.1–5.0	33.0 ($\bar{X}$)	0.07	Red wine Austria	Konakovsky et al. (2011)
	UV-vis λ:	n.d. – 10.8	n.d. – 18.8	2.4–31.8	0–1.1	–	–	n.d.	n.d.	n.d.	4.3–67.0	0.1–0.5	Red wine Italy	Martuscelli et al. (2013)
	254 nm	n.d. – 3.4	n.d. – 6.8	0.8–12.8	0.3–1.2	–	–	n.d.	n.d.	n.d.	3.6–19.5		White wine Italy	
		n.d. – 4.3	n.d. – 10.8	1.4–9.9	0.7–1.1	–	–	n.d. – 1.5	n.d.	n.d. – 1.4	3.7–24.7		Rose wine Italy	
	Fl $\lambda_{ex/em}$: 254/510 nm	2.2–16.2	8.9–37.3	8.3–17.4	–	–	–	–	–	–	27.7–60.9	–	Red wine Italy	Comuzzo et al. (2013)
	Fl $\lambda_{ex/em}$: 320/523 nm	23.1	–	1.5	0.1	–	0.1	0.2	–	n.q.	26.4 ($\bar{X}$)	0.003–0.22	Red wine Portugal	Ramos et al. (2014)
		2.9–8.9	–	0.2–0.3	n.q. – 0.1	–	0.2–0.4	n.q. – 0.2	–	n.q.	4.4–10.0		White wine Portugal	
		15.1	–	2.2	0.0	–	0.3	n.q.	–	n.q.	19.5 ($\bar{X}$)		Rose wine Portugal	
	Fl $\lambda_{ex/em}$: 293/492 nm	Tr-8.1	5.1–11.5	11.4–32.8	1–2.4	n.d.	0.2–1.7	n.d. – 1.2	n.d.	n.d. – 1.27	49.4 ($\bar{X}$)	0.004–0.06	Red wine Italy	Tuberoso et al. (2015)
		n.d.	n.d.	1.5–10.6	1.1–2.5	n.d.	1.1–2.2	n.d. – 1.8	n.d.	n.d.-Tr	16.2 ($\bar{X}$)		White wine Italy	
DEEMM	UV-vis λ:	0.6–12.0	0.5–2.6	3.9–15.4	0.4–0.8	n.d. – 1.1	–	n.d. – 0.1	1.9–3.8	–	10.4–33.5	0.1–0.6	Red wine Spain	Gómez-Alonso et al. (2007)
	280 nm	n.d. – 0.7	n.d. – 5.5	3.9–18.4	0.2–0.3	0.4–1.1	–	n.d. – 0.1	0.3–2.4	–	5.4–26.7		White wine Spain	
		0.5–14.1	0.1–12.4	3.7–48.7	0.1–1.8	–	0.4–36.6	0.1–2.7	–	–	37.3 ($\bar{X}$)	0.1–0.5	Red wine France	Bach et al. (2012)
		n.d. – 6.2	0.1 – 1.4	2.4–25.1	0.2–1.7	1.3–11.8	–	0.1–0.4	–	0.4–9.7	6.9–42.8	–	Red wine Spain	Martínez-Pinilla et al. (2013)
		n.d.	2.6 – 2.8	4.8–5.2	1.3–1.8	n.d.	–	1.5–1.9	n.d.	4.0–4.5	26.4–26.8	0.03–0.1	Red must China	Wang et al. (2014)
		20.4–23.1	6.2–6.5	11.4–2.6	1.6–3.8	n.d.	–	2.3–4.8	n.d. – 2.2	2.6–9.4	86.3–96.1		Red wine China	
AQC	Fl $\lambda_{ex/em}$: 250/395 nm	n.d.	0.8- 0.9	4.2–15.3	0.5–1.0	–	–	–	–	–	5.6–17.0	0.0004–0.027	Red must Spain	Hernández-Orte et al. (2006)
		2.9–4.7	0.6- 0.7	5.7–16.4	0.7–1.5	–	–	–	–	–	11.5–21.7		Red wine Spain	
		0.1–33.0	0.3–10.8	5.0–135	0.3–2.5	–	–	–	–	–	10.0–180.4	0.015–0.050	Red wine Spain	Peña-Gallego et al. (2009)
		n.d. – 0.3	n.q.	0.1–0.3	n.d. – 0.1	n.q. – 0.1	n.d.	n.d.	n.d. – 0.1	n.d. – 0.1	0.1–0.8	0.007–0.026	Red wine vinegar Spain	Ordóñez et al. (2013)
		n.q.	n.d.	n.q. – 0.1	n.d. – 0.0	n.q.	n.d.	n.d.	0.1	n.q.	0.1–0.2		White wine vinegar Spain	

Der. reagent: Derivatization reagent. His: Histamine. Tyr: Tyramine. Put: Putrescine. Cad: Cadaverine. Agm: Agmatine. Met: Methylamine. Phe: Phenylethylamine. Spm: Spermine. Spmd: Spermidine. UV–vis: UV–vis detection. Fl: Fluorescence detection. λ ex/em: λ excitation/emission. n.d.: non detected. n.q: non quantified. –: non determinated. Tr: Traces. $\bar{X}$: Value. OPA: o-phthalaldehyde. AQC: 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. DNS-CL: dansyl chloride. DEEMM: diethyl ethoxymethylenemalonate. LOD: Limit of detection.

Table 2
Chemical properties of biogenic amines standards.

Name	Histamine dihydrochloride	Tyramine hydrochloride	Putrescine dihydrochloride	Cadaverine dihydrochloride	Agmatine sulfate salt	Methylamine hydrochloride	Phenylethylamine hydrochloride	Spermine tetrahydrochloride	Spermidine trihydrochloride
Abbreviation	HIS	TYR	PUT	CAD	AGM	MET	PHE	SPM	SPMD
Purity	≥99.0%	≥98%	≥98%	~98%	≥97.0%	≥99.0%	≥98%	≥99.0%	≥98%
Supplier	Sigma	Sigma	Sigma	Sigma	Sigma	Sigma	Sigma	Sigma	Sigma
Structure	$C_5H_9N_3{\cdot}2HCl$	$C_8H_{11}NO{\cdot}HCl$	$C_4H_{12}N_2{\cdot}2HCl$	$C_5H_{14}N_2{\cdot}2HCl$	$C_5H_{14}N_4{\cdot}H_2SO_4$	$CH_3NH_2{\cdot}HCl$	$C_8H_{11}N{\cdot}HCl$	$C_{10}H_{26}N_4$	$C_7H_{19}N_3{\cdot}3HCl$
Molecular Weight (g/mol)	184.07	173.65	161.07	175.10	228.27	67.52	121.18	348.18	254.63
N° CAS	56-92-8	60−19-5	333−93-7	1476−39-7	2482−00-0	593−51-1	64−04-0	306−67-2	334−50-9
pKa*	pK_1 = 9.8 pK_2 = 6.0	pK = 9.6	pK_1 = 10.8 pK_2 = 9.4	pK_1 = 11.0 pK_2 = 9.9	pK_1 = 12.5	pK = 10.6	pK = 10.0	pK_1 = 11.50 pK_2 = 10.95 pK_3 = 9.79 pK_4 = 8.90	pK_1 = 9.5 pK_2 = 10.8 pK_3 = 11.6
Log Po/w	−0.70	0.68	−0.85	−0.40	−1.18	−0.63	1.39	−0.70	−1.15
Solubility	Easily soluble in cold water, hot water, methanol	Very slightly soluble in cold water	Easily soluble in hot and cold water	Soluble in water, ethanol; slightly soluble in ethyl ether	Water	Soluble in water, ethanol, benzene, and acetone, miscible in ether	Soluble in water and alcohol	Water	Water
Solubility in water	50 mg/mL	50 mg/mL	100 mg/mL	100 mg/mL	50 mg/mL	1080 mg/mL	100 mg/mL	100 mg/mL	100 mg/mL

Ka: acid dissociation constant. Po/w: Partition coefficient (octanol/water). References: Sigma (https://www.sigmaaldrich.com/spain.html); Pubchem (https://pubchem.ncbi.nlm.nih.gov/); Chemicalize (http://www.chemicalize.org); Önal et al. (2013)*.

et al., 2014); alcoholic fermentation (García-Marino et al., 2010; Martínez-Pinilla et al., 2013; Rodriguez-Naranjo et al., 2013; Wang et al., 2014); malolactic fermentation (García-Marino et al., 2010; Martínez-Pinilla et al., 2013; Wang et al., 2014); aging in barrels or tanks (García-Marino et al., 2010; Hernández-Orte et al., 2008); and in a closed bottle (González Marco and Ancín Azpilicueta, 2006; Pérez-Magariño et al., 2013). However, reports focusing on the changes in the concentration of biogenic amines in an opened wine bottle are scarce.

In general, total concentrations of biogenic amines in red wines are higher than in white wines (Konakovsky et al., 2011; Martuscelli et al., 2013). This fact is due to the malolactic fermentation that takes place in all red wines and just in some white wines, converting malic acid into lactic acid and increasing the medium pH. This reaction is performed by lactic acid bacteria, which are the main producers of biogenic amines and, in addition, their decarboxylase enzymes improve their activity with a higher pH (Landete et al., 2007).

High–performance liquid chromatography (HPLC) is the most employed technique for the determination of biogenic amines due to its high resolution, sensitivity and simple preparation (Hernández-Orte et al., 2006). However, it is necessary to use derivatization reagents to improve sensitivity in the analysis (Peña-Gallego et al., 2012). Table 1 shows the different reagents used, together with the detection systems applied and the concentrations of biogenic amines found in grape musts, wines and wine vinegars from different countries. AQC (6–aminoquinolyl–N–hydroxysuccinimidyl carbamate) has been widely employed as a derivatization reagent for the determination of biogenic amines with low detection limits (50–300 fmol) (Callejón et al., 2008; Hernández-Orte et al., 2006; Peña-Gallego et al., 2009).

The existing studies on the field of the biogenic amines profile in finished wines are mainly focused on wines kept in closed bottles. Given that in the restaurant sector wine may be kept in opened bottles, we found interesting to monitor the evolution of biogenic amines in opened bottles along time. The aim of this work was to evaluate the evolution of biogenic amines in different bottled wine samples once opened and submitted to different storage conditions in terms of temperature, kind of stopper and use of vacuum devices, in order to ascertain if these conditions may change the original biogenic amine profile.

2. Materials and methods

2.1. Reagents and standards

The “AccQ–Fluor” Kit supplied by Waters (Milford, MA, USA) consists of 6–aminoquinolyl–N–hydroxysuccinimidyl carbamate reagent (AQC), acetonitrile to dissolve the reagent, and 0.2 mM sodium borate buffer, pH 8.8. The Oasis MCX 1cc (30 mg) Extraction Cartridges resins were acquired from Waters. Biogenic amine standards (Table 2), α–aminobutyric acid (internal standard) (purity ≥99%), calcium disodium EDTA (purity ≥99%) and calcium chloride (purity ≥96%) were obtained from Sigma–Aldrich (Steinheim, Germany). Sodium acetate (purity ≥99%) and sodium hydroxide (purity 98–100.5%) were supplied by Panreac (Castellar del Vallès, Barcelona, Spain), and trihydrate triethylamine (TEA) (purity ≥99%), methanol (HPLC grade) (purity ≥99.8%) and hydrochloric acid 32% (v/v) were acquired from Merck (Darmstadt, Germany). Orthophosphoric acid 85% (v/v) was purchased from Prolabo (Fontenay–sous–bois, France). Ultrapure water was obtained from a Milli–Q water purification system (Millipore, Bedford, MA, USA).

The amine standard solutions (n = 9) were prepared individually by dissolving the pure compounds in methanol, except agmatine and spermine which were dissolved in water. Concentrated solutions of amine standards (500 mg/L) were prepared by diluting the standard solution with water.

Table 3
Evolution of biogenic amine concentrations and pH in standard and high quality red wines and young white wine in different storage conditions.

a)

	Standard quality red wine (n = 12) (Mean concentration (mg/L) ± Standard deviation)											
Biogenic amines	A_0	A_4	A_{10}	B_0	B_4	B_{10}	C_0	C_4	C_{10}	D_0	D_4	D_{10}
Histamine	$16.8^{c} \pm 0.76$	$18.6^{b} \pm 0.76$	$11^{b,c} \pm 1.07$	$15.9^{c} \pm 0.04$	$18.5^{b} \pm 0.94$	$9.82^{b,c} \pm 0.75$	$15.7^{c} \pm 0.14$	$16.5^{b} \pm 0.98$	$11.6^{b,c} \pm 0.28$	$15.1^{c} \pm 0.85$	$18.3^{b} \pm 1.99$	$10.8^{b,c} \pm 0.58$
Agmatine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Methylamine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tyramine	5.99 ± 0.54	5.69 ± 0.02	5.70 ± 0.58	$6.81^{c} \pm 0.00$	6.24 ± 0.30	$5.54^{c} \pm 0.39$	6.10 ± 0.34	5.69 ± 0.11	5.99 ± 0.42	6.09 ± 0.17	5.62 ± 0.16	5.79 ± 0.16
Putrescine	15.6 ± 2.00	13.5 ± 0.00	14.0 ± 0.52	15.3 ± 0.36	15.7 ± 1.15	14.6 ± 0.86	$14.9^{c} \pm 0.11$	14.5 ± 0.71	$14.3^{c} \pm 0.11$	13.3 ± 1.03	15.4 ± 0.06	14.7 ± 0.27
Cadaverine	$0.41^{c} \pm 0.03$	$0.38^{b} \pm 0.00$	$0.30^{b,c} \pm 0.00$	$0.41^{c} \pm 0.01$	$0.42^{b} \pm 0.02$	$0.30^{b,c} \pm 0.01$	$0.42^{c} \pm 0.01$	$0.40^{b} \pm 0.00$	$0.32^{b,c} \pm 0.00$	$0.39^{c} \pm 0.02$	$0.34^{b} \pm 0.00$	$0.32^{b,c} \pm 0.00$
Phenylethyl amine	$0.17^{c} \pm 0.01$	$0.17^{b} \pm 0.00$	$0.24^{b,c} \pm 0.00$	$0.18^{c} \pm 0.01$	$0.17^{b} \pm 0.00$	$0.26^{b,c} \pm 0.02$	$0.16^{c} \pm 0.01$	$0.17^{b} \pm 0.01$	$0.27^{b,c} \pm 0.00$	$0.17^{c} \pm 0.00$	$0.18^{b} \pm 0.01$	$0.26^{b,c} \pm 0.01$
Spermidine	$0.20^{a,c} \pm 0.02$	$0.44^{a,b} \pm 0.00$	$0.48^{b,c} \pm 0.01$	$0.19^{a,c} \pm 0.01$	$0.48^{a} \pm 0.02$	$0.49^{c} \pm 0.04$	$0.18^{a,c} \pm 0.01$	$0.43^{a,b} \pm 0.01$	$0.50^{b,c} \pm 0.00$	$0.21^{a,c0.00}$	$0.45^{a,b} \pm 0.01$	$0.51^{b,c} \pm 0.00$
Spermine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	$39.2^{c} \pm 3.36$	$38.7^{b} \pm 0.72$	$31.7^{b,c} \pm 2.17$	$38.8^{c} \pm 0.34$	$41.5^{b} \pm 2.39$	$31^{b,c} \pm 2.07$	$37.4^{c} \pm 0.39$	$37.7^{b} \pm 0.14$	$33^{b,c} \pm 0.04$	$35.3^{c} \pm 0.37$	$40.3^{b} \pm 2.20$	$32.4^{b,c} \pm 0.71$
pH	3.40	3.41	3.39	3.37	3.36	3.36	3.37	3.38	3.35	3.36	3.36	3.36

b)

	High quality red wine (n = 12). (Mean concentration (mg/L) ± Standard deviation)											
Biogenic amines	A_0	A_4	A_{10}	B_0	B_4	B_{10}	C_0	C_4	C_{10}	D_0	D_4	D_{10}
Histamine	$11.3^{c} \pm 0.25$	9.42 ± 0.89	$7.58^{c} \pm 0.09$	8.83 ± 0.49	$9.48^{b} \pm 0.18$	$7.79^{b} \pm 0.42$	$10.2^{c} \pm 0.64$	$10.3^{b} \pm 0.13$	$7.64^{b,c} \pm 0.06$	9.22 ± 0.75	$11.0^{b} \pm 0.40$	$7.76^{b} \pm 0.36$
Agmatine	$1.26^{a} \pm 0.11$	$2.35^{a,b} \pm 0.07$	$1.28^{b} \pm 0.13$	1.56 ± 0.13	$2.48^{b} \pm 0.31$	$1.35^{b} \pm 0.04$	1.57 ± 0.07	1.48 ± 0.12	1.56 ± 0.03	$2.69^{a} \pm 0.09$	$1.86^{a} \pm 0.18$	2.32 ± 0.14
Methylamine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tyramine	$2.94^{a} \pm 0.37$	$4.64^{a,b} \pm 0.26$	$2.84^{b} \pm 0.11$	2.75 ± 0.16	3.05 ± 0.13	3.89 ± 0.46	$3.45^{a} \pm 0.17$	$4.06^{a,b} \pm 0.05$	$3.20^{b} \pm 0.03$	4.21 ± 0.14	4.09 ± 0.36	3.64 ± 0.14
Putrescine	5.93 ± 0.86	5.16 ± 0.66	4.96 ± 0.03	4.20 ± 0.43	5.17 ± 0.40	4.67 ± 0.09	$5.17^{a} \pm 0.19$	$3.98^{a,b} \pm 0.00$	$5.36^{b} \pm 0.09$	$4.68^{a} \pm 0.01$	$3.61^{a} \pm 0.27$	5.22 ± 0.47
Cadaverine	$0.36^{c} \pm 0.02$	0.34 ± 0.02	$0.30^{c} \pm 0.00$	0.31 ± 0.01	0.35 ± 0.01	0.32 ± 0.01	$0.34^{a,c} \pm 0.00$	$0.31^{a,b} \pm 0.00$	$0.37^{b,c} \pm 0.00$	$0.33^{a,c} \pm 0.00$	$0.32^{a,b} \pm 0.00$	$0.38^{b,c} \pm 0.01$
Phenylethyl amine	0.35 ± 0.03	$0.30^{b} \pm 0.01$	$0.35^{b} \pm 0.00$	$0.30^{a} \pm 0.00$	$0.38^{a,b} \pm 0.00$	$0.31^{b} \pm 0.01$	0.27 ± 0.03	$0.30^{b} \pm 0.00$	$0.35^{b} \pm 0.00$	$0.24^{c} \pm 0.02$	0.32 ± 0.03	$0.36^{c} \pm 0.00$
Spermidine	$0.65^{c} \pm 0.01$	0.56 ± 0.03	$0.55^{c} \pm 0.00$	0.54 ± 0.04	0.63 ± 0.07	0.52 ± 0.00	0.55 ± 0.05	0.50 ± 0.02	0.49 ± 0.02	$0.55^{c} \pm 0.01$	0.51 ± 0.04	$0.45^{c} \pm \pm 0.02$
Spermine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	$22.8^{c} \pm 1.14$	22.8 ± 1.94	$17.9^{c} \pm \pm 0.31$	18.5 ± 1.26	21.5 ± 1.10	18.9 ± 0.93	$21.6^{c} \pm 0.65$	$20.9^{b} \pm 0.02$	$19^{b,c} \pm 0.02$	21.9 ± 0.71	21.7 ± 0.93	20.1 ± 1.10
pH	3.45	3.46	3.46	3.45	3.46	3.44	3.45	3.44	3.44	3.44	3.44	3.45

c)

	Young white wine (n = 12) (Mean concentration (mg/L) ± Standard deviation)											
Biogenic amines	A_0	A_4	A_{10}	B_0	B_4	B_{10}	C_0	C_4	C_{10}	D_0	D_4	D_{10}
Histamine	$1.43^{a,c} \pm 0.08$	$2.81^{a} \pm 0.07$	$2.43^{c} \pm 0.02$	$1.43^{a,c} \pm 0.08$	$2.96^{a} \pm 0.25$	$2.37^{c} \pm 0.07$	$1.43^{a,c} \pm 0.08$	$2.95^{a} \pm 0.05$	$2.41^{c} \pm 0.29$	$1.43^{a,c} \pm 0.08$	$2.92^{a} \pm 0.23$	$2.53^{c} \pm 0.27$
Agmatine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Methylamine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tyramine	$1.73^{c} \pm 0.07$	$1.93^{b} \pm 0.06$	$1.42^{b,c} \pm 0.05$	1.73 ± 0.07	2.01 ± 0.19	1.89 ± 0.02	1.73 ± 0.07	1.58 ± 0.04	1.84 ± 0.22	$1.73^{c} \pm 0.07$	$1.65^{b} \pm 0.01$	$2.14^{b,c} \pm 0.07$
Putrescine	1.19 ± 0.06	1.11 ± 0.03	1.11 ± 0.06	1.19 ± 0.06	1.14 ± 0.03	1.20 ± 0.02	1.19 ± 0.06	1.32 ± 0.03	1.30 ± 0.09	$1.19^{a} \pm 0.06$	$1.44^{a,b} \pm 0.01$	$1.29^{b} \pm 0.04$
Cadaverine	0.25 ± 0.00	0.26 ± 0.00	0.25 ± 0.00	0.25 ± 0.00	$0.26^{b} \pm 0.00$	$0.25^{b} \pm 0.00$	0.25 ± 0.00	$0.27^{b} \pm 0.00$	$0.25^{b} \pm 0.00$	$0.25^{a} \pm 0.00$	$0.27^{a,b} \pm 0.00$	$0.25^{b} \pm 0.00$
Phenylethyl amine	$0.22^{a} \pm 0.00$	$0.26^{a,b} \pm 0.00$	$0.21^{b} \pm 0.01$	$0.22^{a,c} \pm 0.00$	$0.27^{a,b} \pm 0.00$	$0.24^{b,c} \pm 0.00$	$0.22^{a} \pm 0.00$	$0.27^{a,b} \pm 0.00$	$0.22^{b} \pm 0.01$	$0.22^{a} \pm 0.00$	$0.29^{a,b} \pm 0.00$	$0.23^{b} \pm 0.02$
Spermidine	$0.26^{a,c} \pm 0.01$	$0.15^{a,b} \pm 0.01$	$0.20^{b,c} \pm 0.01$	$0.26^{a,c} \pm 0.01$	$0.14^{a,b} \pm 0.00$	$0.16^{b,c} \pm 0.00$	$0.26^{a,c} \pm 0.01$	$0.15^{b,c} \pm 0.01$	$0.19^{b,c} \pm 0.01$	$0.26^{a,c} \pm 0.01$	$0.15^{a} \pm 0.01$	$0.17^{c} \pm 0.01$
Spermine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	5.08 ± 0.08	6.53 ± 0.03	5.62 ± 0.14	5.08 ± 0.08	6.79 ± 0.47	6.12 ± 0.03	5.08 ± 0.08	6.54 ± 0.10	6.21 ± 0.09	5.08 ± 0.08	6.72 ± 0.25	6.63 ± 0.14
pH	3.06	3.00	3.04	3.06	3.03	3.02	3.06	3.01	3.00	3.06	3.01	3.02

Superscript "a" shows significant differences between day 0 and day 4 according to ANOVA test ($p < 0.05$). Superscript "b" shows significant differences between day 4 and day 10 according to ANOVA test ($p < 0.05$). Superscript "c" shows significant differences between day 0 and day 10 according to ANOVA test ($p < 0.05$). n.d.: non detected. Limits of detection (mg/L): histamine: 0.050; agmatine: 0.054; methylamine: 0.057; tyramine: 0.061; putrescine: 0.046; cadaverine: 0.069; phenylethylamine: 0.053; spermidine: 0.027; spermine: 0.070.

2.2. Samples

Three different types of wine were considered as follows: standard quality red wine (up to three months in wood barrel) elaborated with 100% Garnacha grapes from Navarra (Spain), containing 13.5% (v/v) ethanol and pH 3.37; high quality red wine (at least twelve months in wood barrel. DO Rioja 2011) elaborated with 80% Tempranillo grapes and 20% Garnacha tinta and Mazuelo grapes from Rioja (Spain), containing 13.5% (v/v) ethanol and pH 3.45; and bulk young white wine elaborated with a mixture of Garrido fino, Pedro Ximenez and Palomino fino grapes from Sevilla (Spain), containing 11.5% (v/v) ethanol and pH 3.06. Four bottles of each red wine and a container of bulk white wine (5 L) were purchased from local supermarkets in Sevilla (Spain). Then, the container of bulk white wine was divided into four bottles and subsequently stoppered. The variables selected for storage conditions were temperature and kind of stopper. Regarding temperature, wine bottles were maintained at room (25 °C) or refrigerated (4 °C) temperature. Likewise, concerning the kind of stopper, two strategies were applied to stopper the bottles: either they were re-corked or we used a stopper which has a vacuum pump that extracts the air from the bottle (Vacu Vin ®). Thus, samples were coded as A (Room temperature and cork stopper), B (Room temperature and vacuum), C (Refrigeration temperature and cork stopper), and D (Refrigeration temperature and vacuum).

An aliquot of 30 mL was taken from each bottle 0, 4 and 10 days after it was opened, and they were immediately frozen. Ten days were set as the maximum reasonable time for an opened bottle to be consumed, as the sensory qualities are progressively lost due to oxidative degradation and the effects of microorganisms (Silva Ferreira et al., 2003; Bartowsky and Henschke, 2008). The analysis of biogenic amines from each sample was carried out in duplicate, hence a total of 36 wine samples were analysed.

2.3. Samples preparation

Solid–phase extraction (SPE) was performed in order to avoid interferences in wine samples, following the method proposed by Peña-Gallego et al. (2009). The resin employed was Oasis MCX 1 cc (30 mg) Extraction Cartridges resins from Waters. SPE were performed adding 0.6 mL of wine samples into extraction cartridges, they were cleaned with different solutions (2 mL of 10 mM H_3PO_4/MeOH (90:10) solution, 2 mL of 10 mM NaOH/MeOH (70:30) solution and 2 mL of 10 mM $CaCl_2$/MeOH (70:30)) and they were eluted with 1.2 mL of the 100 mM NaOH/MeOH (65:35) solution in a vial containing 100 μL of 1.2 M HCl.

2.4. Determination

Work solutions were prepared prior to the derivatization reaction. Thereby, the mixture was prepared adding 100 μL of internal standard (α–aminobutyric acid at a concentration of 2 mg/L) either to 900 μL of standards (biogenic amines concentrated solutions) or to samples after the extraction.

Determination of biogenic amines was performed by high–-performance liquid chromatography (HPLC) employing AQC as derivatizing reagent, following the procedure previously adapted and validated by our research group (Ordóñez et al., 2013). Thus, 20 μL of work solution was buffered with 60 μL of a 0.2 M solution of sodium borate at pH 8.8, and the derivatization reaction was made by adding 20 μL of the AQC solution.

HPLC analysis was carried out in a Waters equipment consisting of an autosampler injector Waters 717 and a Waters 1525 Binary HPLC pump system controller connected to a fluorescence detector, Waters 474. Data treatment was performed in a Waters Millennium data station. The column was a Luna C18, 5 μm, 250 × 4.6 mm and guard column 4.0 × 3.0 mm from Analytical Phenomenex, (Torrance, CA, USA).

Detection was carried out by fluorescence with excitation at 250 nm and emission at 395 nm. The injection volume was 10 μL and the separation was obtained at a flow rate of 1 mL/min at 65 °C with a gradient program employed by Ordóñez et al. (2013).

Mobile phase A consisted of a 140 mM solution of sodium acetate trihydrate and 17 mM of TEA adjusted to pH 5.05, and mobile phase B was methanol. All mobile phases were filtered through a membrane filter with a mean pore size of 0.45 μm (Millipore, Bedford, MA, USA) prior to use.

The linearity of the method was determined by a regression analysis of the relative area (ratio between peak area of biogenic amines to the peak area of the internal standard) versus the amine concentration. In order to do so, standard solutions of amines in concentrations ranging from 0.02 to 15 mg/L were prepared and analysed in triplicate, and correlation coefficients (R^2) above 0.997 for all the biogenic amines were obtained. According to ICH guidelines (2005), the limits of detection (LD) and quantitation (LQ) were calculated based on the ratio of 3.3 σ/S and 10 σ/S, respectively. Thus, σ is the standard deviation of the response, and S is the slope of the calibration curve. The LD ranged from 0.027 (spermidine) to 0.07 mg/L (spermine) and the LQ were between 0.083 (spermidine) and 0.212 mg/L (spermine). LD of most amines were similar to those reported by other authors who also used AQC (Peña-Gallego et al., 2009; Ordóñez et al., 2013).

2.5. Statistical analysis

All statistical analyses were performed by means of STATISTICA software version 7 (StatSoft, Tulsa, OK, USA). One–way ANOVA was performed to evaluate significant differences between sampling days during the evolution of wine (significance levels $p < 0.05$). Principal Component Analysis (PCA) was carried out as an unsupervised method to visualize the degree of differentiation between the different types of wine samples. Linear Discriminant Analysis (LDA) was carried out to evaluate whether the profiles of biogenic amines were different enough to distinguish between days 0, 4 and 10. Correlation was performed to ascertain the degree of relationship between the different biogenic amines.

3. Results and discussion

The evolution of biogenic amines in opened wine bottles was monitored along time. These bottles were kept under different conditions in terms of temperature, kind of stopper and use of vacuum devices. The period of time considered in this study was 10 days.

3.1. Biogenic amines profile in just opened bottles

The biogenic amine concentrations (mg/L) determined in the different wine samples considered in this study are summarised in Table 3. The total content of biogenic amines ranged from 5.08 to 39.2 mg/L in the set of just opened wine samples. In previous reported studies, the total concentrations of biogenic amines in wines were highly variable from trace to 180 mg/L (Table 1). As expected, the white wine samples presented a significantly lower concentration (Table 3). On the other hand, standard quality red wine samples had twice the concentration of biogenic amines than high quality red wine samples. These data are in accordance with García-Marino et al. (2010) who observed that press red wine had higher amounts of biogenic amines than the free run red wine, as this one is of a higher quality.

The most abundant biogenic amines in the three types of wines were histamine and putrescine, as expected (Table 3). Putrescine is formed by ornithine decarboxylase or arginine deiminase (De Las Rivas et al., 2008; Mangani et al., 2005). Red wines had higher amounts of histamine, putrescine and, in a lesser extent, tyramine than white wine, whilst the concentration of cadaverine was similar in all the samples (Table 3). These results agreed with previous studies (Table 1)

Table 4
Correlation between different biogenic amines in the wine samples.

	Histamine	Agmatine	Tyramine	Putrescine	Cadaverine	Phenylethylamine	Spermidine
Histamine		−0.0318	0.898	0.876	0.863	−0.420	0.364
Agmatine	−0.0317		−0.128	−0.323	0.133	0.698	0.665
Tyramine	0.898	−0.128		0.944	0.767	−0.446	0.287
Putrescine	0.876	−0.323	0.944		0.733	−0.518	0.205
Cadaverine	0.863	0.133	0.767	0.733		−0.259	0.285
Phenylethylamine	−0.420	0.698	−0.446	−0.518	−0.259		0.504
Spermidine	0.364	0.665	0.287	0.205	0.285	0.504	

Significance: $p < 0.05$.

emphasizing the fact that these biogenic amines are mainly formed during the malolactic fermentation (Konakovsky et al., 2011; Mangani et al., 2005), and this could explain the lower amounts of these amines in white wines.

In addition, standard quality red wine presented equal amounts of histamine and putrescine, whilst the rate for high quality red wine was 2:1. These results were in line with those obtained by García-Marino et al. (2010). According to Preti et al. (2016) the histamine concentration was higher in red wines with a lower price. On the contrary, they reported that red wines with a higher price showed a higher amount of putrescine. On the other hand, histamine, putrescine and tyramine showed similar concentrations in white wine, in accordance with other studies (Table 1). In addition, agmatine was only quantified in a low concentration in the high quality red wine. This may be due to the presence of this biogenic amine in grapes, as stated in a previous study (Bauza et al., 2007). Moreover, agmatine is a precursor in one biochemical pathway of the synthesis of putrescine (Landete et al., 2008). Regarding the rest of biogenic amines, such as methylamine, phenylethylamine, spermine and spermidine, they were either not determined or quantified in low concentrations, according to other studies with different types of wines (Henríquez-Aedo et al., 2012; Marcobal et al., 2005; Rodriguez-Naranjo et al., 2013). As reported in previous studies, the low concentrations of volatile amines (phenylethylamine and methylamine) could be due to their decrease during the fermentation process (Ancín-Azpilicueta et al., 2008; Herbert et al., 2005).

3.2. Effect of the storage time and conditions

During the storage of the opened bottles of wine at the different conditions, slight changes were observed in the profile of biogenic amines and the pH (Table 3).

In the standard quality red wine, the total amount of biogenic amines showed a significant trend to decrease along time, regardless of the storage conditions (Table 3a). This trend is mainly observed in the concentration of histamine, and in a lesser extent in cadaverine, which decreased significantly from the fourth to the tenth day in all cases (Table 3a). However, phenylethylamine and spermidine increased significantly along time in all conditions, but their amounts are so low that they do not affect the total concentrations.

Regarding the high quality red wine, some differences were found between the different conditions of storage (Table 3b). Thus, those samples that were not kept in vacuum showed a significant trend to decrease along time, whilst those wines kept in vacuum did not show significant changes in the total concentrations of biogenic amines. These differences are mainly due to tyramine, since this amine decreased significantly from the fourth day of storage in those wines kept in vacuum. In this type of wine, histamine also diminished along time in all conditions. In previous studies, Vidal-Carou et al. (1990) observed that histamine and tyramine concentrations did not increase during wine spoilage in different temperatures of storage. Regarding cadaverine, we can highlight its significant increase from the fourth to the tenth day in those wines kept refrigerated, with or without vacuum.

Unlike red wines, white wine did not show a clear and significant trend in the total content of biogenic amines. Although the concentrations of the different amines are much lower, we can observe a significant increase in histamine concentrations from the fourth day in all conditions, as opposed to red wines (Table 3). Besides, phenylethylamine and spermidine showed an opposite trend. Thus, at the beginning, phenylethylamine increased significantly while spermidine decreased, whereas from the fourth day both trends were reversed. Cadaverine decreased significantly in all conditions from the fourth day, except in the sample kept at room temperature and without vacuum, whose concentrations were constant.

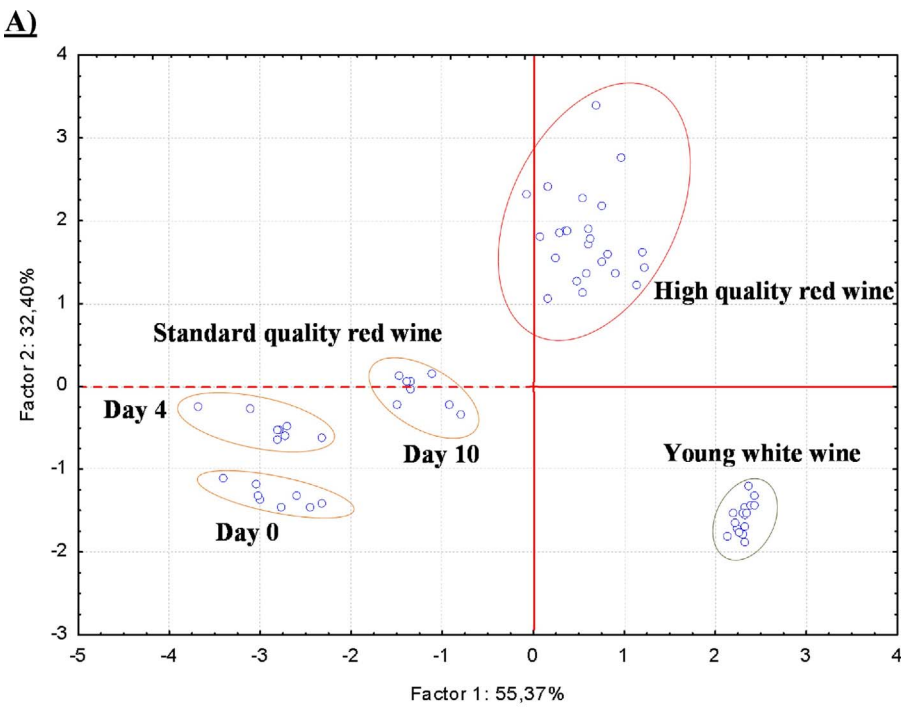
In general, the evolution of biogenic amines in the three types of wine did not show a clear common trend. It should be pointed out that the histamine concentration decreased in both red wines but increased in white wine. This fact is in accordance to Ortega-Heras et al. (2014) who observed that the content of histamine increased about 0.4 mg/L after 6 months in bottle in samples of white wine.

In addition, the different storage conditions employed in this experiment did not affect the biogenic amines profile and the pH. Similarly, most biogenic amines showed just slight changes during their storage at different temperatures in closed red wine bottles (González Marco and Ancín Azpilicueta, 2006).

3.3. Statistical exploration of the data

Considering the concentration of biogenic amines in all the samples, we found a marked positive correlation between histamine, tyramine, putrescine and cadaverine ($r = 0.73–0.94$) (Table 4). These results were similar to those obtained by other authors (Herbert et al., 2005; Konakovsky et al., 2011). Specially, histamine and cadaverine showed a marked positive correlation in the three types of wine; thus, we obtained $r = 0.73$ for white wine, whilst $r = 0.76$ and $r = 0.50$ were obtained for standard and high quality red wine, respectively.

Principal component analysis (PCA) was performed to evaluate whether the profiles of biogenic amines were useful to separate and group the different types of wine selected in this study. Thus, the first two principal components accounted for 87.8% of the total variance. Fig. 1 shows the corresponding scores, and loadings were plotted using the first two principal components. Regarding the loadings of the variables, the first principal component (PC1) is constituted by the positive effect of agmatine and phenylethylamine, while putrescine, tyramine, histamine, cadaverine and spermidine had a negative effect. Thus, the graphic of scores shows that the high quality red wine and the young white wine samples were placed in the right side (positive), unlike the standard quality red wine samples. On the other hand, the positive effect in the second principal components (PC2) is formed by all the biogenic amines, except for putrescine, which was located in the negative section. As shown in Fig. 1(A), high quality red wine samples were placed in the quadrant which was more influenced by agmatine and phenylethylamine. Besides, PCA was not able to group samples of this wine regarding their storage time. Standard quality red wine samples were placed in the quadrant dominated by putrescine. These samples showed a slight trend regarding the storage time. This fact was due to an overall change in the biogenic amines profile, mainly related



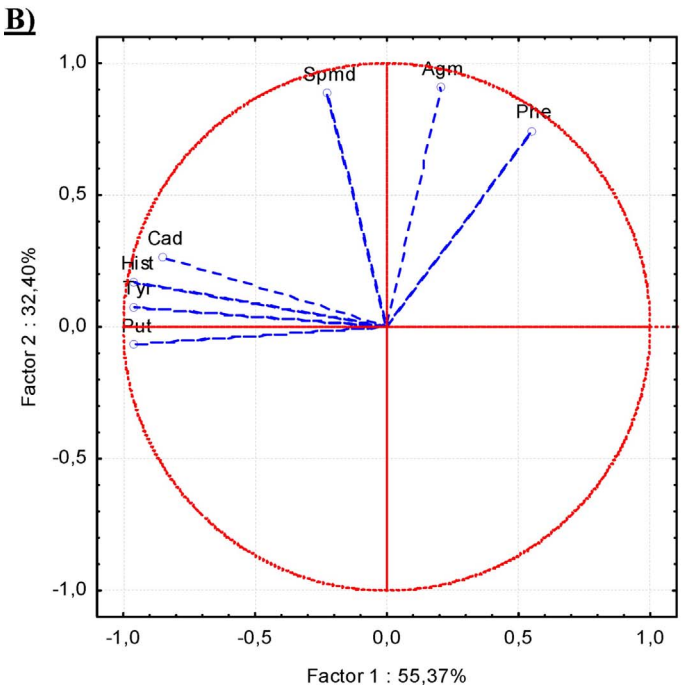


Fig. 1. Graphs of the first two principal components of wine samples. Scores of samples (A) and loadings of variables (B).

to histamine, putrescine, spermidine and agmatine. Finally, young white wine samples were placed in the quadrant which was less related to biogenic amines, and these samples did not show any trend regarding the storage time.

In order to explore differences among samples taking into account the storage time for each wine, another multivariate statistical analysis was applied. Hence, three linear discriminant analyses (LDA) were performed to evaluate whether the concentrations of biogenic amines were different enough to distinguish the samples from days 0, 4 and 10 for the three types of wine under study. A standard analysis was employed for each wine type, using the whole set of biogenic amine concentrations as variables, obtaining a classification matrix of 100% for all cases. Thus, the *Wilk's Lambda* for standard quality red wine, high quality red wine and young white wine were 0.000491, 0.037 and 0.00387, respectively. Besides, *p-value* was 0.000 in all the analyses, showing that the variables selected had power for discrimination. The scatterplots of the canonical scores are shown in Fig. 2. As it can be observed, the biogenic amines profiles were able to separate the samples of the three wines according to their storage time and regardless of storage conditions, although the distances were quite close, especially

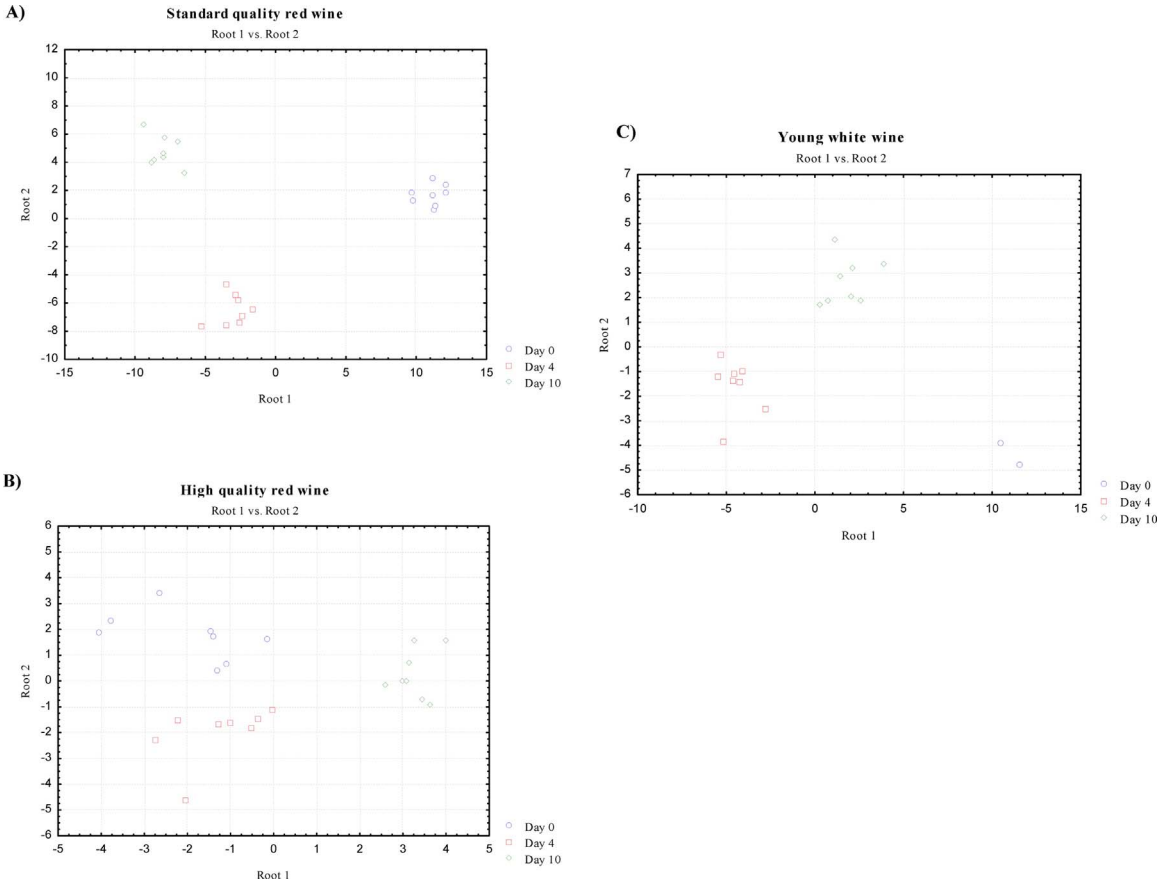


Fig. 2. The scatterplot of canonical scores of standard analysis for standard quality red wine (A), high quality red wine (B) and young white wine (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

in high quality red wine (Fig. 2). Hence, this wine showed to have a higher stability along time. Besides, these results confirmed that the different storage conditions did not produce important changes in the biogenic amines profile.

4. Conclusions

This work concludes that the bottles of wine opened and kept in different storage conditions (temperature and stopper) did not show an important increase in the concentrations of biogenic amines. The biogenic amines profile changed slightly along time in the three types of wines, but the final concentration of these biomolecules in wines was mainly determined by their initial amounts. Histamine, followed by putrescine, were the most abundant biogenic amines, but their concentrations were not enough to cause food poisoning. LDA confirmed that the samples were grouped according to their storage time, regardless of the storage conditions.

In general, these results suggest that the concentrations of biogenic amines in opened wine bottles suffered slight changes during storage. Hence, further microbiology studies, as well as chemical stability analysis, are necessary to determinate which factors affect mainly in the evolution of biogenic amines during storage.

Acknowledgements

We thank the Ministry of Economy and Competitiveness of the Spanish government for its financial support of projects AGL2013–47300–C3–2–R.

References

Ancín-Azpilicueta, C., González-Marco, A., Jiménez-Moreno, N., 2008. Current knowledge about the presence of amines in wine. Crit. Rev. Food Sci. Nutr. 48, 257–275.
Arrieta, M.P., Prats-Moya, M.S., 2012. Free amino acids and biogenic amines in Alicante Monastrell wines. Food Chem. 135, 1511–1519.
Bach, B., Le Quere, S., Vuchot, P., Grinbaumc, M., Barnavon, L., 2012. Validation of a method for the analysis of biogenic amines: histamine instability during wine sample storage. Anal. Chim. Acta 732, 114–119.
Bartowsky, E.J., Henschke, P.A., 2008. Acetic acid bacteria spoilage of bottled red wine-a review. Int. J. Food Microbiol. 125, 60–70.
Bauza, T., Kelly, M.T., Blaise, A., 2007. Study of polyamines and their precursor amino acids in Grenache noir and Syrah grapes and wine of the Rhone Valley. Food Chem. 105 (1), 405–413.
Callejón, R.M., Tesfaye, W., Torija, M.J., Mas, A., Troncoso, A.M., Morales, M.L., 2008. HPLC determination of amino acids with AQC derivatization in vinegars along submerged and surface acetifications and its relation to the microbiota. Eur. Food Res. Technol. 227, 93–102.
Comuzzo, P., Rauhut, D., Werner, M., Lagazio, C., Zironi, R., 2013. A survey on wines from organic viticulture from different European countries. Food Control 34, 274–282.
De Las Rivas, B., González, R., Landete, J.M., Muñoz, R., 2008. Characterization of a second ornithine decarboxylase isolated from Morganella morganii. J. Food Protect. 71, 657–661.
Del Prete, V., Costantini, A., Cecchini, F., Morassut, M., Garcia-Moruno, E., 2009. Occurrence of biogenic amines in wine: the role of grapes. Food Chem. 112 (2), 474–481.
EFSA, 2011. Panel on Biological Hazards (BIOHAZ). Scientific Opinion on risk based control of biogenic amine formation in fermented foods. EFSA J. 9 (10), 2393.
European Commission Regulation, 2005. Commission regulation (EC) No 2073/2005 of november 2005 on microbiological criteria for foodstuffs. Off. J. Eur. Union L 338/1-26.
Gómez-Alonso, S., Hermosín-Gutiérrez, I., García-Romero, E., 2007. Simultaneous HPLC analysis of biogenic amines amino acids, and ammonium ion as aminoenone derivatives in wine and beer samples. J. Agr. Food Chem. 55, 608–613.
García-Marino, M., Trigueros, Á., Escribano-Bailón, T., 2010. Influence of oenological practices on the formation of biogenic amines in quality red wines. J. Food Compos. Anal. 23, 455–462.
González Marco, A., Ancín Azpilicueta, C., 2006. Amine concentrations in wine stored in

bottles at different temperatures. Food Chem. 99, 680–685.
Henríquez-Aedo, K., Vega, M., Prieto-Rodriguez, S., Aranda, M., 2012. Evaluation of biogenic amines content in Chilean reserve varietal wines. Food Chem. Toxicol. 50, 2742–2750.
Herbert, P., Cabrita, M.J., Ratola, N., Laureano, O., Alves, A., 2005. Free amino acids and biogenic amines in wines and musts from the Alentejo region. Evolution of amines during alcoholic fermentation and relationship with variety, sub–region and vintage. J. Food Eng. 66, 315–322.
Hernández-Orte, P., Peña-Gallego, A., Ibarz, M.J., Cacho, J., Ferreira, V., 2006. Determination of the biogenic amines in musts and wines before and after malolactic fermentation using 6–aminoquinolyl–N–hydroxysuccinimidyl carbamate as the derivatizing agent. J. Chromatogr. A 1129, 160–164.
Hernández-Orte, P., Lapeña, A.C., Peña-Gallego, A., Astrain, J., Baron, C., Pardo, I., et al., 2008. Biogenic amine determination in wine fermented in oak barrels: factors affecting formation. Food Res. Int. 41, 697–706.
ICH, 2005. Validation of analytical procedures: test and methodology Q2(R1) In: ICH Guidelines, http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf (Accessed 19 April 2016).
Konakovsky, V., Focke, M., Hoffmann-Sommergruber, K., Schmid, R., Scheiner, O., Moser, P., et al., 2011. Levels of histamine and other biogenic amines in high–quality red wines. Food Addit. Contam. 28 (4), 408–416.
Krymchantowski, A.V., Da Cunha Jevoux, C., 2014. Wine and headache. Headache 54, 967–975.
López, R., Tenorio, C., Gutiérrez, A.R., Garde-Cerdán, T., Garijo, P., González-Arenzana, L., et al., 2012. Elaboration of Tempranillo wines at two different pHs: influence on biogenic amine contents. Food Control 25, 583–590.
Landete, J.M., Ferrer, S., Pardo, I., 2007. Biogenic amine production by lactic acid bacteria, acetic bacteria and yeast isolated from wine. Food Control 18 (12), 1569–1574.
Landete, J.M., Arena, M.E., Pardo, I., Manca de Nadra, M.C., Ferrer, S., 2008. Comparative survey of putrescine production from agmatine deamination in different bacteria. Food Microbiol. 25, 882–887.
Maintz, L., Novak, N., 2007. Histamine and histamine intolerance. Am. J. Clin. Nutr. 85, 1185–1196.
Mangani, S., Guerrini, S., Granchi, L., Vincenzini, M., 2005. Putrescine accumulation in wine: role of Oenococcus oeni. Curr. Microbiol. 51, 6–10.
Marcobal, A., Polo, M.C., Martín-Álvarez, P.J., Moreno-Arribas, M.V., 2005. Biogenic amine content of red Spanish wines: comparison of a direct ELISA and an HPLC method for the determination of histamine in wines. Food Res. Int. 38, 387–394.
Martínez-Pinilla, O., Guadalupe, Z., Hernández, Z., Ayestarán, B., 2013. Amino acids and biogenic amines in red varietal wines The role of grape variety, malolactic fermentation and vintage. Eur. Food Res. Technol. 237, 887–895.
Martuscelli, M., Arfelli, G., Manetta, A.C., Suzzi, G., 2013. Biogenic amines content as a measure of the quality of wines of Abruzzo (Italy). Food Chem. 140, 590–597.
Önal, A., Tekkeli, S.E.K., Önal, C., 2013. A review of the liquid chromatographic methods for the determination of biogenic amines in foods. Food Chem. 138, 509–515.
Ordóñez, J.L., Callejón, R.M., Morales, M.L., García-Parrilla, M.C., 2013. A survey of biogenic amines in vinegars. Food Chem. 141, 2713–2719.
Ortega-Heras, M., Pérez-Magariño, S., Del Villar-Garrachón, V., González-Huerta, C., Moro-Gonzalez, L.C., Guadarrama Rodríguez, A., et al., 2014. Study of the effect of vintage, maturity degree, and irrigation on the amino acid and biogenic amine content of a white wine from the Verdejo variety. J. Sci. Food Agric. 94, 2073–2082.
Pérez-Magariño, S., Ortega-Heras, M., Martínez-Lapuente, L., Guadalupe, Z., Ayestarán, B., 2013. Multivariate analysis for the differentiation of sparkling wines elaborated from autochthonous Spanish grape varieties Volatile compounds, amino acids and biogenic amines. Eur. Food Res. Technol. 236, 827–841.
Panconesi, A., 2008. Alcohol and migraine: trigger factor, consumption, mechanisms. A review. J. Headache Pain 9, 19–27.
Peña-Gallego, A., Peña-Gallego, P., Cacho, J., Ferreira, V., 2009. Biogenic amine determination in wines using solid–phase extraction: a comparative study. J. Chromatogr. A 1216, 3398–3401.
Peña-Gallego, A., Hernandez-Orte, P., Cacho, J., Ferreira, V., 2012. High–performance liquid chromatography analysis of amines in must and wine: a review. Food Rev. Int. 28, 71–96.
Preti, R., Vieri, S., Vinci, G., 2016. Biogenic amine profiles and antioxidant properties of Italian red wines from different price categories. J. Food Compos. Anal. 46, 7–14.
Ramos, R.M., Valente, I.M., Rodrigues, J.A., 2014. Analysis of biogenic amines in wines by salting–out assisted liquid–liquid extraction and high–performance liquid chromatography with fluorimetric detection. Talanta 124, 146–151.
Rodriguez-Naranjo, M.I., Ordóñez, J.L., Callejón, R.M., Cantos-Villar, E., Garcia-Parrilla, M.C., 2013. Melatonin is formed during winemaking at safe levels of biogenic amines. Food Chem. Toxicol. 57, 140–146.
Silva Ferreira, A.C., Hogg, T., Guedes de Pinho, P., 2003. Identification of key odorants related to the typical aroma of oxidation-spoiled white wines. J. Agric. Food Chem. 51, 1377–1381.
Tuberoso, C.I.G., Congiu, F., Serreli, G., Mameli, S., 2015. Determination of dansylated amino acids and biogenic amines in Cannonau and Vermentino wines by HPLC–FLD. Food Chem. 175, 29–35.
Vidal-Carou, M.C., Ambatlle-Espunyes, A., Ulla-Ulla, M.C., Mariné-Font, A., 1990. Histamine and tyramine in spanish wines: their formation during the winemaking process. Am. J. Enol. Viticult. 41, 160–167.
Wang, Y.Q., Ye, D.Q., Zhu, B.Q., Wu, G.F., Duan, C.Q., 2014. Rapid HPLC analysis of amino acids and biogenic amines in wines during fermentation and evaluation of matrix effect. Food Chem. 163, 6–15.

CAPÍTULO IV

Resumen

Este capítulo se centra en el desarrollo y validación de un método de cromatografía líquida para la determinación de metabolitos en muestras de orina, plasma y heces de ratas tras la ingesta de un extracto rico en proantocianidinas. De este modo, para la detección de los analitos se utilizó un espectrómetro de masas de alta resolución (HRMS) que nos permitía obtener información sobre las estructuras de los derivados formados. Además, no se usaron enzimas hidrolíticas, tales como glucuronidasas o sulfatasas, permitiendo identificar metabolitos conjugados específicos. Además, el uso de varios compuestos derivados no comerciales obtenidos por síntesis química permitió una validación y cuantificación más precisa de éstos.

El método analítico fue validado exitosamente para la determinación de un total de 34 metabolitos, incluyendo 3 flavanoles, 4 derivados de valerolactonas y 27 ácidos fenólicos y ácidos aromáticos en tres matrices biológicas (plasma, orina y heces) en términos de especificidad, linealidad, sensibilidad (LD y LQ), precisión (intradía y interdía), recuperación y efecto matriz de acuerdo con los criterios establecidos por EURACHEM (EURACHEM, 2014).

El método se aplicó en muestras de orina, plasma y heces de ratas tras la ingesta de un extracto vino rico en proantocianidinas. El contenido fecal total de derivados de ácidos fenólicos y aromáticos fue de 683±152 nmol, equivalente al 4,6% del extracto de proantocianidina ingerido. Por otro lado, los derivados de ácidos fenólicos y aromáticos fueron los principales compuestos encontrados en las muestras de orina, con una excreción total de 2567 ± 230 nmol, equivalente al 17.3% de la ingesta. En el caso de las muestras de plasma, los valores más altos de concentración máxima (Cmax) fueron para el ácido 3-(3’-hidroxifenil)propiónico (2568 nmol/L), el ácido 3-(fenil)propiónico-3’-sulfato (1405 nmol /L), el ácido 4-hidroxibenzoico (2660 nmol/L) y el ácido hipúrico (433478 nmol/L).

La aplicación del método permitió identificar por primera vez 8 derivados conjugados de fenilvalerolactonas y 6 derivados de ácidos fenilvaléricos en las muestras de orina, plasma y heces tras la ingesta del extracto de semilla de uva rica en proantocianidinas.

Este estudio proporciona una evaluación detallada de la absorción, el metabolismo y el catabolismo del extracto rico en proantocianidinas por las ratas, mostrando la importante participación de la microbiota colónica en su transformación por el organismo. Además, la identificación de nuevos metabolitos procedentes de la degradación microbiana de estos compuestos ayudó a proponer una ruta de degradación colónica para las proantocianidinas.

Artículo 6

Development and validation of an UHPLC-HRMS protocol for the analysis of flavan-3-ol metabolites and catabolites in urine, plasma and feces of rats fed a red wine proanthocyanidin extract.

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Food Chemistry
252 (2018) 49–60

Q1; 6/130 (JCR-ISI, Food Science & Technology, 2016)
Impact Factor: 4.529

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Food Chemistry 252 (2018) 49–60

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Development and validation of an UHPLC-HRMS protocol for the analysis of flavan-3-ol metabolites and catabolites in urine, plasma and feces of rats fed a red wine proanthocyanidin extract

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ARTICLE INFO

Keywords:
Wine proanthocyanidins
UHPLC-HR-MS
Bioavailability
Microbial-derived catabolites
Phenylvalerolactones
Method validation

ABSTRACT

This study developed, optimized and validated an ultra-high-performance liquid chromatography–high-resolution mass spectrometry (UHPLC-HRMS) method to identify and quantify metabolites and microbial-derived catabolites in urine, plasma and feces of rats following ingestion of 50 mg of a red wine proanthocyanidin-rich extract. The method was validated for specificity, linearity, limit of detection (LD) and quantification (LQ), intra-day and inter-day precision, recovery and matrix effects, which were determined for 34 compounds in the three biological matrices. After method validation, three parent flavan-3-ols, four 5-carbon side chain ring fission metabolites, and 27 phenolic acid and aromatic catabolites were quantified in plasma, urine and feces after red wine proanthocyanidin intake. These results establish the value of the UHPLC-HRMS protocol in obtaining a detailed picture of proanthocyanidin metabolites and their microbial-derived catabolites, along with their phase II metabolites, in biological fluids of rat, and potentially in human clinical studies designed to evaluate the bioavailability of dietary flavan-3-ols.

1. Introduction

(Poly)phenols and related compounds are a group of plant-derived secondary metabolites which have been reported to play a key role in the protective effects linked to a plant-based diet rich in fruits, vegetables and their derived products such as cocoa and teas (Del Rio et al., 2013; Granato, Santos, Maciel, & Nunes, 2016). Special attention has focused on the potential health benefits of flavan-3-ols, comprising (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin-3-*O*-gallate and (−)-epigallocatechin-3-*O*-gallate, and their oligomeric forms, the proanthocyanidins (aka condensed tannins) that are formed from the condensation of monomeric units, between two and five units for oligomers and over five units for polymers, differing in their position and configuration of their monomeric linkages, with the dimers B1, B2, B3 and B4 being among those detected most frequently (Crozier, Jaganath, & Clifford, 2006). Flavan-3-ols are commonly present in foodstuffs including cereals, legumes and fruits and vegetables, with especially high levels being found in green tea, red wine, cocoa-based products and apples. Their consumption has been linked to a reduced incidence of chronic diseases such as cardiovascular- and diabetes-related pathologies, as well as neurodegenerative disorders (Crozier et al., 2006; Feliciano, Pritzel, Heiss, & Rodríguez-Mateos, 2015; Rodríguez-Mateos et al., 2014; Zanotti et al., 2015; Zhang & Tsao, 2016).

It is now apparent that it is not the parent (poly)phenols in plant

Abbreviations: UHPLC-HR-MS, ultra-high-performance liquid chromatography coupled to high resolution mass spectrometry; 5C-RFMs, ring fission metabolites with a 5-carbon side chain; MSI, metabolite standards initiative level; MI, metabolite identification level

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https://doi.org/10.1016/j.foodchem.2018.01.083
Received 13 November 2017; Received in revised form 9 January 2018; Accepted 11 January 2018
Available online 12 January 2018
0308-8146/

foods, but their metabolites/colonic catabolites, that exert potential health effects in vivo (Williamson & Clifford, 2017). Unlike other flavonoids, it has been shown that oligomeric and polymeric flavan-3-ols are poorly absorbed in vivo with only dimers, and occasionally trimers, being reported to reach the circulatory system of rats (Serra, Maciá, Romero, Piñol, & Motilva, 2011; Serra et al., 2009; Tsang et al., 2005; Urpi-Sarda, Monagas, Kham, 2009) and humans (Feliciano, Krueger, & Reed, 2015; Sasot et al., 2017; Urpi-Sarda, Monagas, Kham, et al., 2009; Wiese et al., 2015), albeit at very low concentrations. Consequently, the majority of the ingested proanthocyanidins are likely to pass unchanged from the proximal to the distal section of the gastrointestinal tract where they are subjected to the action of the resident microbiota, being converted to a diversity of catabolites, more readily absorbed in situ, such valeric acids and valerolactones and phenolic acids and aromatic compounds (Rodríguez-Mateos et al., 2014; Brindani et al., 2017; Del Rio et al., 2010; Sasot et al., 2017). Since these microbial-derived products and their phase II metabolites may contribute to the health benefits associated to the consumption of flavan-3-ols, it is of importance to determine their levels in biological matrices after the consumption of a flavan-3-ol-rich food.

A number of different analytical methods involving mass spectrometry have been utilised for the analysis of flavan-3-ol metabolites and catabolites in human and animal biological samples after the ingestion of flavan-3-ol rich food products. In some instances samples were subjected to enzyme hydrolysis to deconjugate glucuronide and sulfate moieties prior to analysis (Ding et al., 2013; Saenger, Hubner, & Humpf, 2017). The efficacy of glucuronidase/sulfatase enzyme preparations varies and sulfates are not hydrolysed efficiently and, as a consequence, quantitative estimates are inaccurate (Gasperotti, Masuero, Guella, Mattivi, & Vrhovsek, 2014; Saha et al., 2012). There are only a limited number of investigations in which reliable and validated analytical methods, with appropriate reference compounds, have been utilised for the analysis of metabolites and colon-derived catabolites in biological fluids after a (poly)phenol rich food such as anthocyanins in human urine, plasma and feces (Ferrars et al., 2014) and flavan-3-ols in human urine and plasma (Borges, van der Hooft, & Crozier, 2016; Feliciano, Mecha, Bronze, & Rodriguez-Mateos, 2016; Ottaviani et al., 2016; Sasot et al., 2017) and animal tissues (Goodrich & Neilson, 2014). In the circumstances, sensitive and robust methodologies are needed to accurately quantify metabolites and catabolites derived from flavan-3-ols and other dietary (poly)phenols in biological samples. This would aid not only bioavailability and mode of action studies but also help in the development of reliable biomarkers of (poly)phenol intake in epidemiological studies.

The aim of the current study was to develop and validate an analytical UHPLC-HR-MS method to identify and quantify metabolites and microbial-derived catabolites, using authentic standards. The protocol was then applied to the analysis of urine, plasma and feces of rats following the ingestion of a 50 mg wine proanthocyanidin extract.

2. Experimental

2.1. Chemicals

Formic acid, LC-MS grade acetonitrile, acetic acid, ethyl gallate, (−)-epicatechin, procyanidin B1, procyanidin B2 and the phenolics 3′-hydroxycinnamic acid, ferulic acid, 3-(3′-methoxy-4′-hydroxyphenyl) propionic acid (dihydroferulic acid), 3-(3′-hydroxy-4′-methoxyphenyl) propionic acid (dihydroisoferulic acid), 3-(3′-hydroxyphenyl)propionic acid, 3-(4′-hydroxyphenyl)propionic acid, 3-phenyllactic acid, 3′-hydroxy-4′-methoxyphenylacetic acid (isohomovanillic acid), 3′-methoxy-4′-hydroxyphenylacetic acid (homovanillic acid), 3-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4′-hydroxymandelic acid, 1,3,5-trihydroxyphenol (phloroglucinol) and hippuric acid were purchased from Sigma-Aldrich (Poole, Dorset, U.K.). Ferulic acid-4′-*O*-glucuronide, ferulic acid-4′-sulfate, coumaric acid-3′-*O*-glucuronide, caffeic acid-3′-*O*-glucuronide, isoferulic acid-3′-*O*-glucuronide, 3-(3′-hydroxyphenyl) propionic acid-4′-*O*-glucuronide (dihydrocaffeic acid-4′-*O*-glucuronide), 3-(4′-hydroxyphenyl)propionic acid-3′-*O*-glucuronide (dihydrocaffeic acid-3′-*O*-glucuronide), 3-(3′-methoxyphenyl)propionic acid-4′-*O*-glucuronide (dihydroferulic acid-4′-*O*-glucuronide), 3-(3′-methoxyphenyl)propionic-acid-4′-sulfate (dihydroferulic acid-4′-sulfate), 3-(phenyl)propionic acid-3′-sulfate and 3-(phenyl)propionic acid-3′-*O*-glucuronide were kindly provided by Denis Barron (Nestle Research Center, Lausanne, Switzerland) and Gary Williamson (School of Food Science and Nutrition, University of Leeds, UK). 4′-Hydroxyhippuric acid was obtained from Bachem (UK) Ltd (St Helens, UK). Standard compounds of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 5-(3′-hydroxyphenyl)-γ-valerolactone, 5-(phenyl)-γ-valerolactone-3′-sulfate and 5-(phenyl)-γ-valerolactone-3′-*O*-glucuronide were synthesized according to Curti et al. (Curti et al., 2015) and Brindani et al. (Brindani et al., 2017). They are catalogued on the standards sharing platform FoodComEx (www.foodcomex.org).

2.2. Isolation of a red wine proanthocyanidin extract

A red wine proanthocyanidin extract was obtained from a young, unfinded Cabernet Sauvignon wine from the Coonawarra region of South Australia that had not been stored in barrels and so did not contain any oak-derived proanthocyanidins. The tannin isolation and purification method was that of Bindon and Smith (Bindon & Smith, 2013) with the following modifications. Wine was added directly to a slurry of Amberlite FPX66 resin (Dow Chemical Company, Spring House, PA, USA) prepared according to the manufacturer's instructions, and reconstituted in 12% v/v ethanol to allow adsorption of phenolics. The wine and resin slurry was then filtered through glass wool, and the aqueous fraction discarded. The resin was then washed with Milli-Q water, and the phenolics were eluted in 70% (v/v) acetone containing 0.05% v/v trifluoroacetic acid (TFA) followed by pure acetone. Acetone was removed under reduced pressure at 35 °C and then methanol was added to a final concentration of 50% (v/v) methanol, 0.05% v/v TFA and loaded onto a glass column packed with 500 mL of Toyopearl HW-40C (Sigma, Castle Hill, NSW, Australia) which had been equilibrated with the loading solvent. The column was washed with 2.5 L 50% (v/v) methanol, 0.05% v/v TFA, and the eluant discarded. Proanthocyanidins were eluted using 800 mL of 70% (v/v) acetone containing 0.05% (v/v) TFA. The acetone was removed under reduced pressure at 35 °C, after which the remaining aqueous fraction partitioned three times against half volumes of ethyl acetate which were discarded. The remaining traces of ethyl acetate were removed from the aqueous phase under reduced pressure at 35 °C. The aqueous extract was then frozen in liquid nitrogen, lyophilised and recovered as a fine powder that was stored under nitrogen prior to use.

2.3. Animal feeds

Male Sprague-Dawley rats (Janvier, Le Genest-St-Isle, France) weighing 200 ± 15 g were housed in individual stainless-steel metabolic cages in a controlled temperature environment (23 ± 1 °C), subjected to a 12 h light/dark cycle with free access to both tap water and diet. The animals were handled in compliance with European Union rules and according to the guidelines of the NIH and the Committee on Animal Care at the University of Montpellier (France).

Prior to supplementation, the rats were fasted overnight and received by gavage 50 mg of a red wine proanthocyanidins extract in distilled water (1 mL/rat). At 24 h after gavage, three rats were anesthetized with an intraperitoneal injection of pentobarbital (60 g L^{-1} at a dosage of 60 mg kg^{-1} body), and blood samples were collected in heparinized tubes by cardiac puncture. Plasma was prepared by centrifugation at 4000 rpm for 10 min at 4 °C, and then stored at −80 °C prior analysis. Similarly, urine and feces were collected for a 24 h after

gavage and immediately frozen at −80 °C. In order to obtain baseline values control rats received 1 mL distilled water by gavage.

2.4. Analysis of red wine proanthocyanidin extract

Methanolic aliquots of the red wine proanthocyanidin extract were analysed using an Exactive Orbitrap mass spectrometer (Thermo Scientific, San José, CA, USA) linked to an UHPLC pump, a PDA detector scanning from 200 to 600 nm, and an autosampler operating at 4 °C (Dionex Ultimate 3000 RS, Thermo Corporation). Chromatographic separation was achieved using a 250 × 4.6 mm, 5 μm Develosil Diol 100 Å column (Phenomenex, Cheshire, UK) with chromatographic conditions described by Robbins et al. (2009).

After passing through the flow cell of the PDA detector the column eluate was split and 0.8 mL min^{-1} directed to a FP-2020 fluorescence detector (Jasco, Easton, MD, USA) and 0.2 mL min^{-1} to the UHPLC-HR-MS operating in negative ionization mode. The capillary temperature was 300 °C, sheath gas was 60 units, auxiliary gas was 60 units, sweep gas was 5 units and spray voltage was 4 kV. Data acquisition and processing were carried out using Xcalibur 3.0 software (Thermo Scientific, San José, CA, USA). Proanthocyanidins were identified on the basis of their exact mass and retention time when standards were available and quantified in (−)-epicatechin equivalents using fluorescence peak areas, after which estimates were adjusted to account for the reduced fluorescence response for the proanthocyanidin oligomers and polymers (Robbins et al., 2009).

2.5. Processing of urine, plasma and feces

Plasma samples were defrosted, vortexed and extracted according to Ludwig et al. (2015). Briefly, 400 μL aliquots were mixed with 1 mL of 2% formic acid in acetonitrile. The samples were then vortexed for 1 min and ultrasonicated for 10 min. After centrifugation at 16,100*g* for 10 min, supernatants were reduced to dryness in vacuo using a Speedvac concentrator (Eppendorf, Hamburg, Germany) and resuspended in 100 μL of methanol:water:formic acid (50:50:0.1, v/v/v) which was centrifuged at 16,100*g* for 10 min and 5 μL aliquots of the supernatant analysed by UHPLC-HR-MS.

Feces were extracted using a previous described method (Serra et al., 2009) with some modifications. To 100 mg of freeze-dried feces, 50 μL of ascorbic acid 1% and 100 μL of phosphoric acid 4% were added. The mixture was spiked with 1 μg of ethyl gallate as an internal standard. The samples were first extracted with 800 μL of water/methanol/phosphoric acid 4% (94/4.5/1.5, v/v/v) using a sonicator for 30 s (Digital Sonifier model S-150D ultrasonic cell disruptor, Branson, Teltow, Germany) and maintained in ice to avoid heat. The supernatant was decanted and the pellet re-extracted three times with 500 μL of the same solvent, as described above. The three supernatants were combined, a 1 mL aliquot mixed with 1 mL of 4% phosphoric acid and loaded onto OASIS HLB cartridges (3 mL, 60 mg) previously conditioned with 1 mL of methanol and 1 mL of 0.2% acetic acid. Column was washed with 1 mL 4% phosphoric acid and 1 mL of 0.2% acetic acid. The retained compounds were eluted with 2 × 1 mL acetone/MilliQ water/acetic acid (70/29.5/0.5, v/v/v). The eluate was reduced to dryness using a Speedvac concentrator (Eppendorf, Germany) and resuspended in 25 μL of acidified methanol (1% formic acid) to which 225 μL of 0.1% aqueous formic acid was added. The resuspended extracts were centrifuged at 16,000*g* for 10 min at 4 °C in a 0.2 μm Micro−spin Eppendorf filter (Alltech Associates Applied Sciences, Lancashire, UK) prior to analysis by UHPLC-HR-MS.

2.6. UHPLC-HR-MS analysis of urine, plasma and feces

Separation was performed on a 150 × 4.6 mm i.d. 2.6 μm Kinetex Phenyl-Hexyl 100 A column (Phenomenex, Torrance, CA,) maintained at 40 °C. The mobile phase was pumped at a flow rate of 0.4 mL min^{-1} with 60 min gradient of 3–50% of 0.1% formic acid methanol in 0.1% aqueous formic acid. After passing through the flow cell of the PDA detector the column eluate went directly to an Exactive Orbitrap mass spectrometer (Thermo Scientific, San José, CA) fitted with a heated electrospray ionization probe (HESI) operating in negative ionization mode. Full scans were recorder in a *m/z* range from 100 to 1800 with a resolution of 50,000 Hz and with a full AGC target of 100,000 charges, using 2 microscans. Analyses were also based on scans with in-source collision-induced dissociation (CID) at 25.0 eV. MS experiment conditions with HESI in negative ionization mode were: (i) capillary temperature was 275 °C, the heater temperature was 100 °C, the sheath gas was 70 units, the auxiliary gas was 10 units, and the spray voltage was 4.0 kV.

Prior to analyses, the Exactive Orbitrap MS was externally calibrated using ready-to-use calibration mixtures (Pierce ESI Negative Ion Calibration Solution and Pierce LQT ESI Positive Ion Calibration Solution) (Thermo Scientific). Quality control samples (QC) were applied to assess and ensure the analytical process. The QC samples, composed of identical aliquots of representative pools of the urine, plasma and fecal samples, were injected regularly throughout the run. Data acquisition and processing were carried out using Xcalibur 3.0 software (Thermo Scientific, San José, CA).

Targeted identifications of (−)-epicatechin metabolites, phenylvalerolactones and other phenolics were achieved by comparing the exact mass and the retention time with available standards. Quantification of metabolites and phenolic catabolites were carried out by selecting the theoretical exact mass of the molecular ion by reference to standard curves prepared in diluted urine, plasma or feces.

2.7. Method validation

The analytical method was validated for specificity, linearity, limit of detection (LD), limit of quantification (LQ), intra-day and inter-day precision and recovery, as well as matrix effects, according to Eurachem guideline (Magnusson & Örnemark, 2014).

Linearity was assessed for 34 compounds (Fig. 1) by preparing individual stock solutions in methanol or dimethyl sulfoxide. These solutions were diluted and pooled to obtain standard solutions at a concentration of 100 μmol/L in 1% of formic acid in methanol. Working solutions were prepared with a concentration ranging from 0.20 nmol L^{-1} to 140 μmol/L for linear calibration curves, depending on each compound. For calibration curves reference compounds were prepared in methanol/formic acid (99:1, v/v) diluted baseline urine, plasma or feces. A minimum of 9 calibration levels were used and a linear regression function was fitted to each compound.

The sensitivity of the method was calculated as the limited of detection (LD) and limit of quantification (LQ) of each compound in the plasma, urine and fecal matrices. The LD was determined as the concentration corresponding to 3 times the signal-to-noise ratio (S/N), while LQ was calculated as the concentration corresponding to 10 times the signal-to-noise ratio (S/N).

The intra-day precision was checked by injecting two different concentrations, one near the LQ (L) and the other five times the LQ (H), in each matrix five times in succession. The inter-day precision was evaluated in five different working sessions over a period of 7 days using the same procedure described above for the intra-day precision. The results were expressed as relative standard deviation (RSD%) and the acceptance criteria was that RSD% < 15%.

The recovery was calculated by using two concentrations of each reference compound in the same manner as described in the preceding paragraph with diluted plasma and feces being spiked with all the standards at two concentrations and were submitted to extraction in duplicate (samples spiked before extraction). These samples were also submitted to extraction and injected directly (blank samples) or spiked with a mixture of analytes in order to have a final concentration (samples spiked after extraction). Urine samples were spiked with

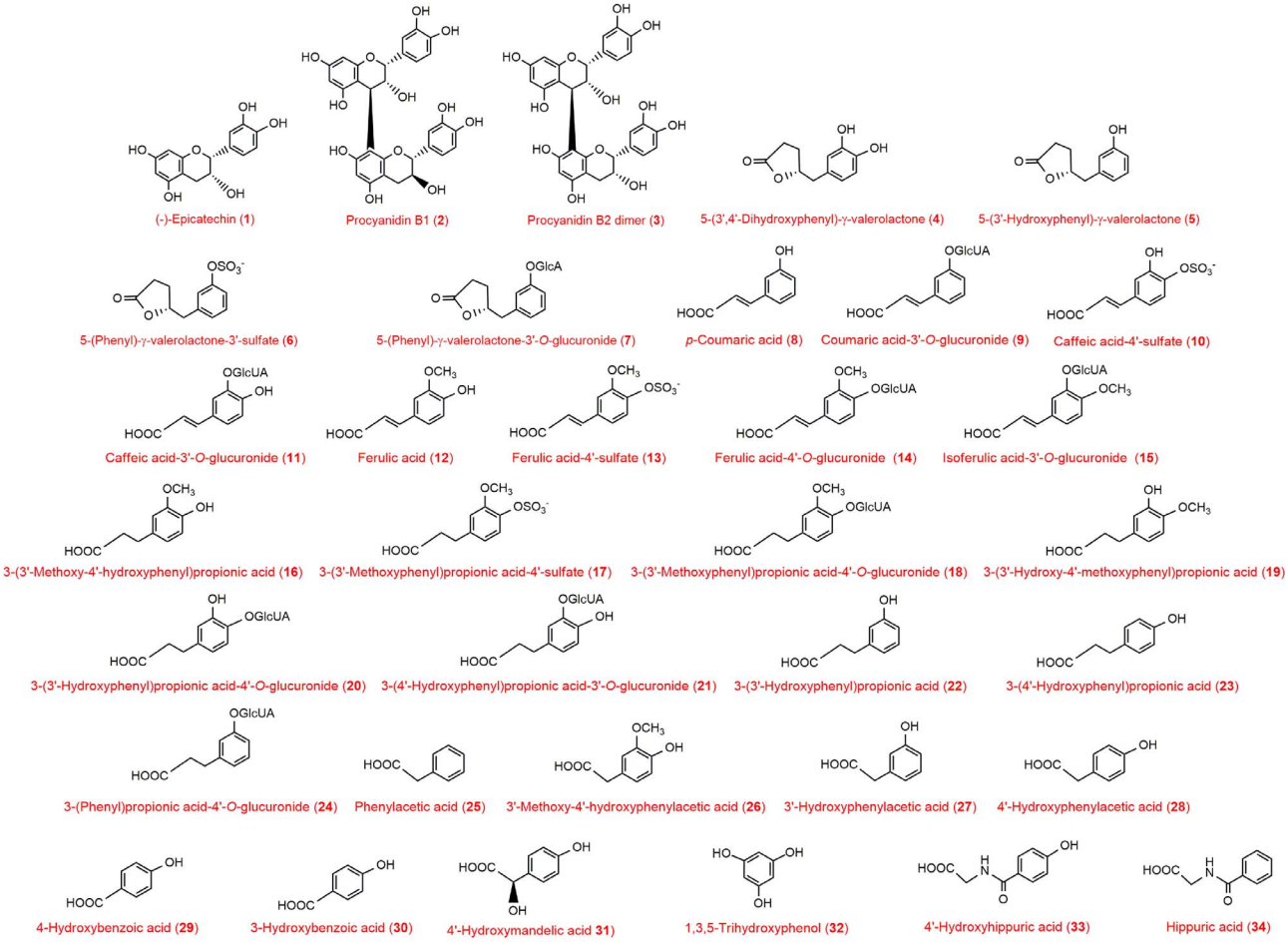

Fig. 1. Chemical structures of the 34 compounds validated in this study.

standards before and after centrifugation.

The recovery for each analyte was calculated as the ratio between the area of samples spiked before and after extraction minus the area of blank samples according to the following formula:

$$R = \frac{\text{Area of samples spiked before extraction} - \text{Area of blank samples}}{\text{Area of samples spiked after extraction} - \text{Area of blank samples}} \times 100$$

The results were expressed as recovery rate.

Matrix effects (ME) were evaluated by comparing the slope of calibration curves prepared in urine, plasma or feces after extraction and the standard curves prepared in methanol with 1% formic acid. The results were expressed as percentages.

Further, specificity was assessed as ppm deviation comparing mass error between the predicted *m/z* and observed *m/z* (Acceptance Criteria for Confirmation of Identity of Chemicals Residues using Exact Mass Data for the FDA Foods and Veterinary Medicine Program, 2015). In addition, the retention time of each analyte was compared in blank solvent (methanol) and different matrices (baseline urine, plasma and feces) previously spiked with standards.

2.8. Statistical analysis

Analyses were performed in triplicate for method validation. Data are reported as mean ± standard error (SE). Peak plasma concentrations (C_{max}) and time to reach C_{max} (T_{max}) values were calculated using the PKSolver add-in software for Microsoft Excel (Zhang, Huo, Zhou, & Xie, 2010).

3. Results and discussion

3.1. Flavan-3-ols in the red wine proanthocyanidin extract

The 50 mg red wine proanthocyanidin extract fed to rats contained the monomers (−)-epicatechin (0.8 μmol) and (+)-catechin (0.7 μmol), procyanidin dimers (4.6 μmol), prodelphinidin dimer (0.32 μmol), procyanidin-*O*-gallate dimers (0.07 μmol), procyanidin trimers (0.3 μmol), prodelphinidin trimers (0.15 μmol), procyanidin tetramers (0.10 μmol), together with small amounts of prodelphinidin tetramers (0.05 μmol), procyanidin pentamers (0.05 μmol), prodelphinidin pentamers (0.02 μmol), procyanidin hexamers (0.02 μmol), heptamers (0.01 μmol), octamers (0.008 μmol), nonamers (0.008 μmol) and decamers (0.003 μmol). The total amount of flavan-3-ols ingested by gavage by each rat was, therefore, 7.2 μmol of which 1.5 μmol was monomers and 5.7 μmol oligomeric and polymeric proanthocyanidins.

3.2. Validation of the method for the analysis of flavan-3-ol-derived compounds in plasma, urine and feces of rats

The UHPLC-HR-MS method was validated for the analysis of potential compounds of interest in using baseline urine, plasma and feces. The validation parameters studied were the response linearity, sensitivity (LD and LQ), intra-day and inter-day precision, recovery and matrix effects (Tables 1–3).

3.2.1. Linearity, limit of detection and limits of quantification

The analytes showed a good linear relation between the tested concentration of standards and the response of the detector over the

Table 1
Parameters for validation and quantification of metabolites in rat urine. Linear calibration curve parameters correspond to slope, intercept and R^2. Limit of detection (LD), limit of quantification (LQ) and linearity are expressed in nmol L^{-1}. Intra-day and inter-day precision are expressed as relative standard deviation (%). Recovery and matrix effect are presented in percentages.

Rt (min)	Metabolites	Slope	Intercept	R^2	LD	LQ	Linearity	Intra-day precision		Inter-day precision		Recovery		Matrix effect
								(H)	(L)	(H)	(L)	(H)	(L)	
	SREMs													
28.3	(−)-Epicatechin	9251	−90,316	0.9938	1.1	3.4	3.4–86,480	0.3	2.7	11.4	11.6	100	97	7.8
21.8	Procyanidin B1	6078	−8,555,667	0.9901	1.7	5.6	5.6–86,630	3.1	3.3	12.5	14.6	97	95	−12.9
29.4	Procyanidin B2	6290	−7,608,830	0.9934	1.7	5.6	5.6–86,630	3.0	5.6	13.6	14.7	98	95	−18.2
	5C-RFMs													
	5-(Dihydroxyphenyl)-γ-valerolactones													
28.8	5-(3′.4′-Dihydroxyphenyl)- γ-valerolactone	4420	−3,234,964	0.9932	290.0	870.0	870–50,000	2.5	5.0	13.5	13.4	99	96	−15.0
	5-(Hydroxyphenyl)-γ-valerolactones													
39.5	5-(3′-Hydroxyphenyl)-γ-valerolactone	317	−199,893	0.9930	270.0	810.0	810–50,000	4.7	5.6	6.9	8.0	96	99	10.8
36.0	5-(Phenyl)-γ-valerolactone-3′-sulfate	10,899	−2,156,894	0.9985	1.0	3.3	3.3–50,000	1.5	1.5	5.3	5.0	96	99	11.6
32.3	5-(Phenyl)-γ-valerolactone-3′-*O*-glucuronide	2169	719,610	0.9973	5.0	16.7	16.7–50,000	1.8	2.3	5.8	5.1	97	98	9.5
	Phenylpropanoid acid derivatives													
37.7	3′-Hydroxycinnamic acid	3469	9,796,075	0.9943	29.9	99.6	99.6–153,340	3.7	5.2	12.9	10.9	100	97	14.5
28.2	Coumaric acid-3′-*O*-glucuronide	10,878	−3,275,086	0.9939	1.0	3.0	3.0–147,460	0.3	3.6	4.7	8.8	98	96	−0.8
26.7	Caffeic acid-4′-sulfate	6818	6,443,699	0.9996	1.5	5.0	5.0–96,530	1.4	6.0	3.8	6.1	99	98	−0.1
26.5	Caffeic acid-3′-*O*-glucuronide	7555	−155,726	0.9933	3.0	10.0	10–140,820	0.8	2.8	7.5	8.6	100	97	−5.7
38.0	Ferulic acid	254	−785,330	0.9976	20.0	60.0	60–129,500	5.4	4.8	9.3	8.4	99	98	−12.9
30.8	Ferulic acid-4′-sulfate	14,411	3,009,994	0.9979	1.4	4.7	4.7–183,150	1.6	2.6	3.4	1.9	100	103	−13.9
26.3	Ferulic acid-4′-*O*-glucuronide	5733	−7,663,148	0.9958	4.2	14.0	14–135,470	1.4	3.1	10.2	10.6	99	100	−8.7
33.3	Isoferulic acid-3′-*O*-glucuronide	9092	−8,297,713	0.9975	3.0	10.0	10–135,470	0.8	2.0	9.0	10.8	99	98	−8.5
	Phenylpropionic acid derivatives													
33.7	3-(3′-Methoxy-4′-hydroxyphenyl) propionic acid	197	−302,809	0.9958	54.0	180.0	189–128,160	4.9	7.0	10.9	6.5	100	99	−5.6
28.8	3-(3′-Methoxyphenyl)propionic acid-4′-sulfate	10,928	−54,654	0.9957	1.4	4.7	4.7–90,900	1.1	1.8	4.2	3.0	101	98	−7.3
27.4	3-(3′-Methoxyphenyl)propionic acid-4′-*O*-glucuronide	7606	−10,624,457	0.9939	5.0	16.0	16–134,730	0.9	2.4	10.3	11.1	95	98	−2.8
37.6	3-(3′-Hydroxy-4′-methoxyphenyl) propionic acid	48	95,683	0.9908	150.2	500.6	501–12,816	3.5	5.8	11.5	7.1	99	100	−18.8
22.4	3-(3′-Hydroxyphenyl)propionic acid-4′-*O*-glucuronide	6435	−6,239,695	0.9975	1.1	3.7	3.7–140,020	2.1	2.8	9.2	9.9	97	96	2.0
23.7	3-(4′-Hydroxyphenyl)propionic acid-3′-*O*-glucuronide	4848	−8,709,275	0.9949	4.4	14.7	14.7–140,020	1.8	3.9	6.9	8.8	100	97	8.2
32.1	3-(3′-Hydroxyphenyl)propionic acid	2281	5,813,488	0.9927	23.7	79.0	79–151,470	3.7	4.9	12.4	8.0	98	99	0.8
28.4	3-(4′-Hydroxyphenyl)propionic acid	14,623	−4,129,236	0.9968	6.0	20.0	20–151,470	0.2	1.5	3.2	8.0	99	97	−14.4
26.6	3-(Phenyl)propionic acid-3′-*O*-glucuronide	8542	2,637,232	0.9919	1.1	3.7	3.7–146,590	0.7	2.8	7.7	9.3	98	100	−3.4
	Phenylacetic acid derivatives													
33.1	Phenylacetic acid	6	2279	0.9985	440.0	1440.0	1440–185,130	2.9	2.7	6.4	3.8	100	99	−13.5
25.3	3′-Methoxy-4′-hydroxyphenylacetic acid	61	-150,770	0.9935	350.0	1070.0	1070–138,080	7.3	10.4	13.4	7.6	100	98	−14.4
24.2	3′-Hydroxyphenylacetic acid	281	−511,389	0.9985	461.8	1540.0	1540–82,760	5.2	7.4	9.0	6.4	101	99	−13.7
20.2	4′-Hydroxyphenylacetic acid	20	-29,300	0.9991	430.0	1300.0	1300–165,520	2.7	5.6	9.4	13.8	100	98	−12.8
	Benzoic acid derivatives													
18.3	4-Hydroxybenzoic acid	604	1,211,689	0.9943	21.4	71.3	71.3–182,450	2.2	5.4	7.2	9.5	100	99	−1.7
22.5	3-Hydroxybenzoic acid	625	−421,626	0.9965	10.8	36.0	36–182,450	2.5	5.4	11.9	14.0	99	98	−15.6
	Hydroxycarboxylic acid derivatives													
6.9	4′-Hydroxymandelic acid	2746	1,317,816	0.9982	17.5	58.0	58–149,670	4.8	3.0	13.2	9.7	100	99	−13.8
	Benzenetriol derivatives													
7.6	1,3,5-Trihydroxybenzene (Phloroglucinol)	11	119,205	0.9954	15.6	52.0	52–199,960	3.5	4.8	10.8	14.2	99	101	−24.8
	Benzoylglycine derivatives													
12.8	4′-Hydroxyhippuric acid	6859	11,486,041	0.9962	1.0	3.0	3.0–128,840	5.3	4.9	14.4	11.3	100	98	12.0
21.7	Hippuric acid	8235	45,119,664	0.9906	1.5	5.0	5.0–140,410	4.5	6.5	13.3	13.8	102	100	−10.9

Rt: Retention time. R^2: coefficient of determination. H: high concentration of standard spiked (5 × LQ). L: Low concentration of standard spiked (1 × LQ).

Table 2
Parameters for validation and quantification of metabolites in rat plasma extracts. Linear calibration curve parameters correspond to slope, intercept and R^2. Limit of detection (LD), limit of quantification (LQ) and linearity are expressed in nmol L^{-1}. Intra-day and Inter-day precision are expressed as relative standard deviation (%). Recovery and matrix effects are presented in percentages.

Rt (min)	Metabolites	Slope	Intercept	R^2	LD	LQ	Linearity	Intra-day precision		Inter-day precision		Recovery		Matrix effect
								(H)	(L)	(H)	(L)	(H)	(L)	
	SREMs													
28.3	(−)-Epicatechin	4172	23,034,976	0.9912	3.4	3.6	3.6–172,970	2.3	1.4	10.4	5.2	100	97	4.1
21.8	Procyanidin B1	1710	−1,303,217	0.9946	1.4	4.8	4.8–86,630	2.7	2.9	2.4	1.4	88	78	4.8
29.4	Procyanidin B2	1887	−602,547	0.9947	1.4	4.8	4.8–86,630	1.2	2.1	5.0	3.1	93	80	5.0
	5C-RFMs													
	5-(Dihydroxyphenyl)-γ-valerolactones													
28.8	5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	4444	545,296	0.9909	310.0	940.0	940–50,000	1.7	3.5	5.1	6.8	93	87	−14.5
	5-(Hydroxyphenyl)-γ-valerolactones													
39.5	5-(3′-Hydroxyphenyl)-γ-valerolactone	237	−90,301	0.9918	340.0	1020.0	1020–50,000	2.8	7.2	11.4	6.6	74	71	−17.4
36.0	5-(Phenyl)-γ-valerolactone-3′-sulfate	9210	5,874,667	0.9952	1.1	3.7	3.7–100,000	3.6	3.9	6.0	7.1	95	92	−5.6
32.3	5-(Phenyl)-γ-valerolactone-3′-*O*-glucuronide	1848	2,788,132	0.9926	4.7	15.7	15.7–100,000	1.4	2.3	4.3	6.2	92	85	−6.7
	Phenylpropanoid acid derivatives													
37.7	3′-Hydroxycinnamic acid	2582	903,317	0.9973	37.5	125.0	125–306,680	2.5	2.5	5.9	4.4	91	92	−14.8
28.2	Coumaric acid-3′-*O*-glucuronide	11,109	6,823,448	0.9964	0.9	3.0	3.0–147,460	1.0	1.5	5.2	1.8	74	73	−3.8
26.7	Caffeic acid-4′-sulfate	6699	18,729,550	0.9919	1.3	4.3	4.3–96,530	3.3	3.7	6.8	7.6	88	87	−1.6
26.5	Caffeic acid-3′-*O*-glucuronide	8247	−843,437	0.9978	2.5	8.3	8.3–70,410	1.5	1.6	6.4	2.6	76	72	2.3
38.0	Ferulic acid	251	−184,815	0.9994	15.0	46.0	46–129,500	4.0	2.6	6.2	4.5	92	87	−0.6
30.8	Ferulic acid-4′-sulfate	14,692	33,167,357	0.9964	1.4	4.7	4.7–181,810	3.6	3.1	6.1	6.6	92	84	−12.2
26.3	Ferulic acid-4′-*O*-glucuronide	5675	2,434,301	0.9917	5.4	18.0	18–135,470	1.1	1.2	3.7	0.7	74	70	−6.6
33.3	Isoferulic acid-3′-*O*-glucuronide	3944	−10,902,053	0.9959	2.7	9.0	9–135,470	1.2	0.7	6.9	2.3	84	76	8.5
	Phenylpropionic acid derivatives													
33.7	3-(3′-Methoxy-4′-hydroxyphenyl) propionic acid	178	−793,704	0.9983	66.0	200.0	200–256,320	5.0	2.6	8.5	4.9	84	90	−14.5
28.8	3-(3′-Methoxyphenyl)propionic acid-4′-sulfate	10,286	41,679,735	0.9922	1.1	3.7	3.7–181,810	3.5	3.5	7.7	8.1	92	89	−12.7
27.4	3-(3′-Methoxyphenyl)propionic acid-4′-*O*-glucuronide	3200	−40,683	0.9997	5.0	16.0	16–134,730	1.4	1.5	5.9	1.9	81	80	12.4
37.6	3-(3′-Hydroxy-4′-methoxyphenyl) propionic acid	46	101,635	0.9962	300.0	1000.0	1000–256,320	3.3	3.9	7.6	4.2	96	88	11.6
22.4	3-(3′-Hydroxyphenyl)propionic acid-4′-*O*-glucuronide	6969	−1,757,621	0.9978	1.5	5.0	5–70,010	1.2	0.9	5.2	2.5	77	74	10.5
23.7	3-(4′-Hydroxyphenyl)propionic acid-3′-*O*-glucuronide	4702	−1,779,985	0.9972	3.2	10.7	10.7–70,010	0.7	1.4	3.4	2.6	77	74	5.0
32.1	3-(3′-Hydroxyphenyl)propionic acid	1886	1,922,653	0.9953	12.0	40.0	40–302,930	3.4	2.6	8.8	3.2	93	90	0.4
28.4	3-(4′-Hydroxyphenyl)propionic acid	13,301	14,052,941	0.9933	6.0	20.0	20–302,930	2.7	1.3	5.0	2.9	99	96	−4.9
26.6	3-(Phenyl)propionic acid-3′-*O*-glucuronide	8716	7,079,464	0.9968	1.0	3.3	3.3–146,590	1.5	0.7	7.4	3.7	79	78	−5.8
	Phenylacetic acid derivatives													
33.1	Phenylacetic acid	6	19,416	0.9980	300.0	1000.0	1000–185,130	6.4	9.7	5.2	10.0	91	89	−8.1
25.3	3′-Methoxy-4′-hydroxyphenylacetic acid	49	−193,721	0.9985	710.0	2160.0	2160–276,170	2.6	4.5	9.7	6.1	92	90	−8.5
24.2	3′-Hydroxyphenylacetic acid	357	66,923	0.9967	290.0	967.0	967–165,520	3.1	3.3	7.0	3.3	85	92	−0.9
20.2	4′-Hydroxyphenylacetic acid	23	−155,371	0.9966	851.0	2580.0	2580–331,040	2.7	11.9	8.2	11.9	95	93	−2.7
	Benzoic acid derivatives													
18.3	4-Hydroxybenzoic acid	666	617,947	0.9927	10.0	36.0	36–182,450	3.4	3.5	6.0	3.7	84	85	−11.0
22.5	3-Hydroxybenzoic acid	690	−1,995,831	0.9955	10.8	36.0	36–364,900	2.7	3.4	6.9	3.1	89	89	-6.7
	Hydroxycarboxylic acid derivatives													
6.9	4′-Hydroxymandelic acid	2737	−1,151,863	0.9981	17.5	58.0	58–149,670	2.3	3.3	7.2	4.1	85	82	−14.1
	Benzenetriol derivatives													
7.6	1,3,5-Trihydroxybenzene (Phloroglucinol)	14	-19,475	0.9934	15.6	52.0	52–399,930	1.9	4.2	8.7	7.0	88	87	−9.0
	Benzoylglycine derivatives													
12.8	4′-Hydroxyhippuric acid	5338	−19,805,412	0.9962	13.0	40.0	40–257,670	3.0	2.7	9.4	4.3	98	87	−12.9
21.7	Hippuric acid	8032	10,936,012	0.9989	10.0	30.0	30–280,820	2.4	2.0	5.6	5.1	101	106	−13.1

Rt: Retention time. R^2: coefficient of determination. H: high concentration of standard spiked (5× LQ). L: Low concentration of standard spiked (1× LQ).

Table 3
Parameters for validation and quantification of metabolites in rat fecal extracts. Linear calibration curve parameters correspond to slope, intercept and R^2. Limit of detection (LD), limit of quantification (LQ) and linearity are expressed in nmol L^{-1}. Intra-day and Inter-day precision are expressed as relative standard deviation (%). Recovery and matrix effects are presented in percentages.

Rt (min)	Metabolites	Slope	Intercept	R^2	LD	LQ	Linearity	Intra-day precision		Inter-day precision		Recovery		Matrix effect
								(H)	(L)	(H)	(L)	(H)	(L)	
	SREMs													
28.3	(−)-Epicatechin	9053	23,773,793	0.9915	1.1	3.4	3.4–86,480	1.7	3.8	4.8	7.9	85	77	5.5
21.8	Procyanidin B1	6138	1,368,614	0.9958	1.7	5.6	5.6–86,630	1.0	4.2	6.3	9.8	< 20	< 20	−12.0
29.4	Procyanidin B2	6215	2,746,542	0.9954	1.7	5.6	5.6–86,630	1.2	4.1	6.5	9.3	< 20	< 20	−19.1
	5C-RFMs													
	5-(Dihydroxyphenyl)-γ-valerolactones													
28.8	5-(3′,4′-Dihydroxyphenyl)- γ-valerolactone	4735	4,019,320	0.9905	620.0	1890.0	1890–50,000	2.3	6.2	8.6	8.8	92	92	−9.0
	5-(Hydroxyphenyl)-γ-valerolactones													
39.5	5-(3′-Hydroxyphenyl)-γ-valerolactone	286	−56,357	0.9926	670.0	2040.0	2040–50,000	2.8	9.0	12.3	14.0	75	71	−0.1
36.0	5-(Phenyl)-γ-valerolactone-3′-sulfate	9239	−48,174	0.9952	1.4	4.7	4.7–50,000	1.2	3.1	8.4	9.7	74	71	−5.3
32.3	5-(Phenyl)-γ-valerolactone-3′-*O*-glucuronide	2084	698,687	0.9982	10.0	33.0	33–50,000	1.2	5.1	3.3	8.4	72	70	5.2
	Phenylpropanoid acid derivatives													
37.7	3′-Hydroxycinnamic acid	2862	2,611,258	0.9960	36.0	120.0	120–153,340	4.4	2.5	5.6	12.8	92	89	−5.6
28.2	Coumaric acid-3′-*O*-glucuronide	11,149	8,751,981	0.9925	0.9	3.0	3.0–147,460	1.1	2.1	5.8	7.8	68	76	1.6
26.7	Caffeic acid-4′-sulfate	6209	11,812,429	0.9907	2.4	8.0	8.0–96,530	0.7	4.4	10.6	7.2	71	68	−8.7
26.5	Caffeic acid-3′-*O*-glucuronide	8206	12,460,959	0.9906	2.8	9.3	9.3–140,820	2.3	2.8	6.8	9.0	75	73	2.4
38.0	Ferulic acid	235	−510,494	0.9946	33.0	101.0	101–129,500	5.6	3.8	9.5	10.9	78	79	−19.6
30.8	Ferulic acid-4′-sulfate	13,439	4,737,719	0.9988	24.0	80.0	80–183,150	1.7	3.3	10.9	10.1	72	69	−19.7
26.3	Ferulic acid-4′-*O*-glucuronide	5842	1,657,891	0.9905	4.2	14.0	14–135,470	1.3	2.4	5.7	8.0	75	70	−7.0
33.3	Isoferulic acid-3′-*O*-glucuronide	9841	8,631,060	0.9931	3.0	10.0	10–135,470	2.1	2.6	6.3	7.5	79	76	−1.0
	Phenylpropionic acid derivatives													
33.7	3-(3′-Methoxy-4′-hydroxyphenyl) propionic acid	174	−1,317,871	0.9952	132.0	401.0	401–128,160	6.9	2.6	9.3	10.8	77	73	−16.6
28.8	3-(3′-Methoxyphenyl)propionic acid-4′-sulfate	10,030	10,551,601	0.9904	2.0	6.6	6.6–90,900	1.2	1.6	10.7	8.2	70	69	−14.9
27.4	3-(3′-Methoxyphenyl)propionic acid-4′-*O*-glucuronide	7680	740,827	0.9987	6.0	20.0	20–134,730	1.1	2.7	5.9	7.9	78	75	−1.9
37.6	3-(3′-Hydroxy-4′-methoxyphenyl) propionic acid	47	678,340	0.9938	150.0	500.0	500–128,160	2.9	1.7	11.1	13.2	89	87	−20.7
22.4	3-(3′-Hydroxyphenyl)propionic acid-4′-*O*-glucuronide	6547	9,605,718	0.9912	1.5	5.0	5.0–140,020	1.6	2.2	7.4	9.0	78	78	3.8
23.7	3-(4′-Hydroxyphenyl)propionic acid-3′-*O*-glucuronide	4607	4,640,615	0.9910	3.2	10.7	10.7–140,020	1.3	3.5	8.1	10.3	73	76	2.8
32.1	3-(3′-Hydroxyphenyl)propionic acid	2209	−11,735,662	0.9903	78.0	260.0	260–151,470	1.9	1.9	12.6	14.4	88	84	−2.4
28.4	3-(4′-Hydroxyphenyl)propionic acid	16,276	25,070,840	0.9968	12.0	40.0	40–151,470	1.5	2.9	5.0	7.0	87	86	−4.8
26.6	3-(Phenyl)propionic acid-3′-*O*-glucuronide	8669	9,127,210	0.9919	1.4	4.7	4.7–146,590	2.2	2.7	7.0	8.2	72	69	−1.9
	Phenylacetic acid derivatives													
33.1	Phenylacetic acid	5	4562	0.9958	440.0	1440.0	1440–185,130	4.5	9.7	9.3	12.1	70	73	−15.7
25.3	3′-Methoxy-4′-hydroxyphenylacetic acid	55	−357,965	0.9961	933.0	3110.0	3110–138,080	2.5	4.2	8.6	10.5	80	78	−22.4
24.2	3′-Hydroxyphenylacetic acid	242	281,325	0.9975	851.0	2590.0	2590–165,520	4.9	3.2	13.7	13.8	80	77	−25.7
20.2	4′-Hydroxyphenylacetic acid	19	27,435	0.9992	850.0	2580.0	2580–165,520	0.9	6.8	7.5	7.7	82	82	−17.8
	Benzoic acid derivatives													
18.3	4-Hydroxybenzoic acid	604	−435,867	0.9967	32.0	106.7	106.7–182,450	3.8	2.7	9.8	12.8	85	81	−1.7
22.5	3-Hydroxybenzoic acid	608	496,390	0.9950	32.0	106.7	106.7–182,450	4.1	3.7	10.1	13.2	82	81	−17.8
	Hydroxycarboxylic acid derivatives													
6.9	4′-Hydroxymandelic acid	3151	−298,632	0.9981	17.5	58.0	58–149,670	2.4	1.9	10.0	11.2	73	70	−1.1
	Benzenetriol derivatives													
7.6	1,3,5-Trihydroxybenzene (Phloroglucinol)	13	52,701	0.9987	11.6	38.7	38.7–199,960	4.1	5.8	9.9	10.1	85	81	−9.8
	Benzoylglycine derivatives													
12.8	4′-Hydroxyhippuric acid	5491	6,383,213	0.9951	17.0	50.0	50–128,840	2.3	1.4	4.4	5.5	91	86	−10.4
21.7	Hippuric acid	8102	−27,307,549	0.9964	40.0	120.0	120–140,410	1.5	1.3	6.2	8.6	86	80	−12.3

Rt: Retention time. R^2: coefficient of determination. H: high concentration of standard spiked (5 × LQ). L: Low concentration of standard spiked (1 × LQ).

range 3.0 to 199,960 nmol L^{-1}, 3.0 to 399,930 nmol L^{-1} and 3.0 to 199,960 nmol L^{-1} in urine, plasma and feces, respectively (Tables 1–3). Thus, the coefficient of determination (R^2) was > 0.99 in the regression analysis of all the analytes in the three matrices.

The LDs and LQs were similar for the three matrices. The LDs ranged from 1.0 to 462 nmol L^{-1} and the LQs from 3.0 to 1540 nM in urine (Table 1). For plasma samples, the LDs ranged from 0.9 to 710 nmol L^{-1} and the LQs from 3.0 and 2160 nmol L^{-1} (Table 2). Finally, LDs ranged from 0.9 to 933 nmol L^{-1} and LQs were between 3.0 and 3110 nmol L^{-1} in feces (Table 3).

3.2.2. Precision and accuracy

The intra-day precision of urine samples, expressed as the relative standard deviation (% RSD), was less than 15%, ranging from 0.2% for 3-(4′-hydroxyphenyl)propionic acid to 10.4% for 3′-methoxy-4′-hydroxyphenylacetic acid (Table 1). The intra-day precision with plasma samples varied between 0.7% and 11.9% for 3-(4′-hydroxyphenyl) propionic acid-3′-*O*-glucuronide and 4′-hydroxyphenylacetic, respectively (Table 2). The intra-day precision obtained with fecal samples ranged from 0.7% for caffeic acid-4′-sulfate to 9.7% for phenylacetic acid (Table 3). Regarding inter-day precision, the values ranged from 0.7 to 10.4, 1.9 to 14.7 and 3.3 to 14.4 for plasma, urine and feces, respectively (Tables 1–3). These data are in accordance to the values proposed by the AOAC guide (RSD < 15%) (AOAC, 1993).

3.2.3. Recovery and matrix effects

Recoveries ranged from 70 to 110% with the different matrices. Thus, recovery in urine samples ranged from 95% to 102%, while plasma was between 70 and 106% (Table 1 and 2). In most instances, the recoveries in feces were slightly lower than in plasma and urine. It is of note that the recovery of procyanidin B1 and B2 in feces were less than 20% (Table 3). This could be due to the reverse-phase sorbent of OASIS HLB cartridges retaining these compounds (Xiao et al., 2017). Sulfated metabolites, which are highly hydrophilic, showed a recovery rate close to 70% in feces, in accordance with values reported by Feliciano et al. (2016).

With regard to specificity, as shown in Table 4, the ppm deviations obtained were < 5 ppm in all instances and are therefore considered as an acceptable level of mass accuracy.

Matrix effects were similar in urine and plasma and slightly higher in feces, with an average of −5.7, −4.2 and −8.3, respectively (Tables 1–3). Thus, there was slight ion suppression in all three matrices as noted previously (Feliciano et al., 2016; Saenger et al., 2017). Although some compounds had a higher matrix effect in feces, most had a matrix effect of ± 20%.

3.3. Method application

3.3.1. Quantitative analysis of flavan-3-ol metabolites and catabolites excreted in urine

Three parent flavan-3-ol compounds, four 5C-RFMs, and 27 phenolic and aromatic acids, were quantified in urine collected 0–24 h after ingestion of 50 mg of the red wine proanthocyanidin extract. These data are present in Table 5 together with the percentage of excretion of the parent flavan-3-ols, 5C-RFMs and phenolic acid catabolites with respect to the total amount of ingested flavan-3-ol monomers and oligomers.

Trace levels of the unmetabolised (−)-epicatechin and the B1 and B2 procyanidins were detected in urine with no excretion of other proanthocyanidin oligomers and polymers. The principal 5C-RFMs was 5-(phenyl)-γ-valerolactone-3′-sulfate (33.7 nmol) followed by 5-(phenyl)-γ-valerolactone-3′-*O*-glucuronide (11 nmol). The total urinary excretion of SREMs was 44.8 ± 0.6 nmol which corresponds to a 0.3% of flavan-3-ol monomer intake. A total of 44.8 ± 0.6 nmol of 5C-RFMs were excreted which, if derived exclusively from flavan-3-ol monomers, would be equivalent to 3% of intake. This, however, is almost certainly an overestimate as some the 5C-RFMs will also be products of some degree of proanthocyanidin breakdown in the colon (Xiao et al., 2017). If in the unlikely event of the ingested proanthocyanidins being fully broken down to monomer units, they could yield 14.8 μmol of 5C-RFMs, so the 44.8 nmol excretion of 5C-RFMs would represent a 0.3% recovery.

The main phenolic acids and aromatic compounds excreted were ferulic acid-4′-sulfate (503 nmol), 3-(3′-methoxy-4′-hydroxyphenyl) propionic acid (97 nmol), 3-(3′-methoxyphenyl)propionic acid-4′-*O*-sulfate (106 nmol), 3-(3′-hydroxy-4′-methoxyphenyl)propionic acid (123 nmol), 3-(3′-hydroxyphenyl)propionic acid (150 nmol), 3-(phenyl) propionic acid-3′-sulfate (595 nmol), 4′-hydroxyphenylacetic acid (178 nmol) and hippuric acid (275 nmol) (Table 5). Overall the phenolic acids and aromatic compounds were the major products derived from the proanthocyanidin extract with the excretion of 2567 ± 230 nmol being equivalent to 17.3% of intake. The data highlight in detail the importance of the colonic microbiota in the transformation of proanthocyanidins as alluded to in earlier studies (Goodrich and Neilson, 2014; Sasot et al., 2017; Ulaszewska et al., 2016; Urpi-Sarda, Monagas, Khan, et al., 2009; Xiao et al., 2017). Furthermore, the presence of substantial quantities of 5C-RFMs is in line with investigations which observed increasing amounts of these compounds in urine after intake of [^{14}C](−)-epicatechin by humans (Borges, Ottaviani, van der Hooft, Schroeter, & Crozier, 2017; Ottaviani et al., 2016) and rats (Borges et al., 2016), as well as after the consumption of flavan-3-ols in tea (Brindani et al., 2017) and cocoa powder by humans (Llorach et al., 2013; Urpi-Sarda, Monagas, Khan, et al., 2009).

3.3.2. Quantitative analysis of flavan-3-ol metabolites and catabolites in plasma

Tables 5 also showed the parameters of the quantified metabolites in rat plasma after the gavage of 50 mg of the red wine proanthocyanidin extract. One hour after intake, indicating absorption in the proximal gastrointestinal tract, (−)-epicatechin (M1) and procyanidins B1 (M2) and B2 (M3) reached C_{max} values of 14.3 and 4.2 and 3.6 nmol L^{-1}, respectively. This is in agreement with previous reports on the presence of (−)-epicatechin and procyanidin dimers in plasma of rodents after ingestion of procyanidins (Baba, Osakabe, Natsume, & Terao, 2002; Serra et al., 2009, 2011; Shoji et al., 2006; Tsang et al., 2005). Overall the proanthocyanidins per se had very limited bioavailability as only trace amounts of dimers accumulated in plasma and no trimers to decamers were detected.

There were 5C-RFMs and more phenolic and aromatic catabolites in plasma, which had delayed 6–24 h T_{max} values (Table 5), indicating that they were colonic in origin formed by microbiota-mediated transformations of the flavan-3-ols in the wine proanthocyanidin extract. Some, but not all, of these products had undergone further post-absorption phase II metabolism. Those with the highest C_{max} values were 3-(3′-hydroxyphenyl)propionic acid (2568 nmol L^{-1}), 3-(phenyl)propionic acid-3′-sulfate (1405 nmol L^{-1}), 4-hydroxybenzoic acid (2660 nmol L^{-1}) and, most notably, hippuric acid (433,478 nmol L^{-1}) (Table 5).

3.3.3. Quantitative analysis of flavan-3-ol metabolites and catabolites in feces

Table 5 also show the quantities of metabolites detected in rat feces 0–24 h after intake of the 50 mg wine proanthocyanidin extract. Traces of (−)-epicatechin (0.01 nmol) were detected but the ingested proanthocyanidins were absent. Three 5C-RFMs were detected but in low amounts totalling 0.16 ± 0.03 nmol equivalents to 0.001% of the ingested proanthocyanidin extract (Table 5). More substantial amounts of phenolic acids and aromatic derivatives were detected in the 0–24 h feces. The main constituents were ferulic acid (63 nmol), ferulic acid-4′-sulfate (78 nmol), 3-(3′-hydroxy-4′-methoxyphenyl)propionic acid (35 nmol), 3′-hydroxyphenylacetic acid (228 nmol), 4′-hydroxyphenylacetic acid (77 nmol), and 4-hydroxybenzoic acid (52 nmol).

Table 4
HPLC-HRMS-characteristics of the validated metabolites: parent flavan-3-ols, and the 5 carbon side chain ring fission metabolites (5C-RFMs) phenyl-γ-valerolactones and phenolic and aromatic acids.

Rt (min)	Flavan3-ols and Metabolites	Chemical Formula [*m/z*]-	Experimental mass [*m/z*]-	δ (ppm)	Fragments low collision energy [*m/z*]-	Location[a]	MSI MI level[a]
	Parent flavan-3-ols						
28.3	(−)-Epicatechin	C15H13O6	289.0708	0.46	245.0808	U,P,F	1
21.8	Procyanidin B1	C30H25O12	577.1348	0.51	425.0889; 289.0725	U,P	1
29.4	Procyanidin B2	C30H25O12	577.1348	0.51	425.0889; 289.0725	U,P	1
	5C-RFMs						
	5-(Dihydroxyphenyl)-γ-valerolactone derivatives						
28.8	5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	C11H11O4	207.0649	−3.38	No fragment	F	1
	5-(Hydroxyphenyl)-γ-valerolactone derivatives						
39.5	5-(3′-Hydroxyphenyl)-γ-valerolactone	C11H11O3	191.0702	2.09	No fragment	F	1
36.0	5-(Phenyl)-γ-valerolactone-3′-sulfate	C11H11O6S	271.0271	0.36	191.0702	U,P,F	1
32.3	5-(Phenyl)-γ-valerolactone-3′-*O*-glucuronide	C17H19O9	367.1021	−0.27	191.0702	U,P	1
	Phenolic and aromatic Acids Catabolites						
	Phenylpropanoid acid derivatives						
37.7	3′-Hydroxycinnamic acid	C9H7O3	163.0378	4.18	119.0481	U	1
28.2	Coumaric acid-3′-*O*-glucuronide	C15H15O9	339.0712	−0.41	163.0386	U,F	1
26.5	Caffeic acid-3′-*O*-glucuronide	C15H15O10	355.0659	0.20	179.0339	U	1
38.0	Ferulic acid	C10H9O4	193.0492	1.73	134.0354	U,F	1
30.8	Ferulic acid-4′-sulfate	C10H9O7S	273.0064	−0.18	193.0494	U,P,F	1
26.3	Ferulic acid-4′-*O*-glucuronide	C16H17O10	369.0812	1.14	193.0495	U,F	1
33.3	Isoferulic acid-3′-*O*-glucuronide	C16H17O10	369.0812	1.14	193.0494	U	1
	Phenylpropionic acid derivatives						
33.7	3-(3′-Methoxy-4′-hydroxyphenyl)propionic acid	C10H11O4	195.0648	1.97	136.0512	U,F	1
28.8	3-(3′-Methoxyphenyl)propionic acid-4′-sulfate	C10H11O7S	275.0218	0.72	195.0652	U,P,F	1
27.4	3-(3′-Methoxyphenyl)propionic acid-4′-*O*-glucuronide	C16H19O10	371.0963	2.62	195.0652	U,F	1
37.6	3-(3′-Hydroxy-4′-methoxyphenyl)propionic acid	C10H11O4	195.0648	1.97	136.0516	U,F	1
22.4	3-(3′-Hydroxyphenyl)propionic acid-4′-*O*-glucuronide	C15H17O10	357.0816	−0.31	181.0491	U,F	1
23.7	3-(4′-Hydroxyphenyl)propionic acid-3′-*O*-glucuronide	C15H17O10	357.0816	−0.31	181.0491	U,F	1
32.1	3-(3′-Hydroxyphenyl)propionic acid	C9H9O3	165.0536	4.18	121.0639	U,P	1
28.4	3-(4′-Hydroxyphenyl)propionic acid	C9H9O3	165.0536	4.18	121.0639	U,P	1
28.2	3-(Phenyl)propionic acid-3′-sulfate	C9H9O6S	245.0108	2.59	165.0540	U,P	1
26.6	3-(Phenyl)propionic acid-3′-*O*-glucuronide	C15H17O9	341.0869	−0.56	165.0542	U,P	1
30.0	3-Phenyllactic acid	C9H10O3	165.0536	4.18	119.0483	U,P	1
	Phenylacetic acid derivatives						
20.7	3′-Hydroxy-4′-methoxyphenylacetic acid	C9H9O4	181.0490	2.95	137.0593	U,F	1
25.3	3′-Methoxy-4′-hydroxyphenylacetic acid	C9H9O4	181.0490	2.95	137.0593	U,F	1
24.2	3′-Hydroxyphenylacetic acid	C8H7O3	151.0383	3.97	107.0486	U,P,F	1
20.2	4′-Hydroxyphenylacetic acid	C8H7O3	151.0384	3.31	108.0438	U,P,F	1
	Benzoic acid derivatives						
18.3	4-Hydroxybenzoic acid	C7H5O3	137.0227	4.37	93.0327	U,P,F	1
22.5	3-Hydroxybenzoic acid	C7H5O3	137.0227	4.37	93.0327	P	1
	Hydroxycarboxylic acid derivatives						
6.9	4′-Hydroxymandelic acid	C8H7O4	167.0333	2.99	121.0279	U,P,F	1
	Benzenetriol derivatives						
7.6	1,3,5-Trihydroxybenzene (Phloroglucinol)	C6H5O3	125.0228	−3.70	No fragment	U	1
	Benzoylglycine derivatives						
12.8	4′-Hydroxyhippuric acid	C9H8NO4	194.0442	2.57	No fragment	U,P,F	1
21.7	Hippuric acid	C9H8NO3	178.0489	5.05	No fragment	U,P	1

[1]Summer, L. W.; Amberg, A.; Barrett, D.; Beale, M. H.; Beger, R.; Daykin, C. A.; Fan, T. W.; Fiehn, O.; Goldagre, R.; Griffin, J. L. Proposed minimum reporting standards for chemical analysis. Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). Metabolomics 2007, 3, 211–221.

[a] Metabolite standards initiative (MSI) metabolite identification (MI) levels[1]. Reference compounds were available for all compounds identified at MSI MI level 1.

The 0–24 h fecal content of phenolic acids and aromatic derivatives was 683 ± 152 nmol, equivalent to 4.6% of the ingested proanthocyanidin extract (Table 5). The presence of several phase II metabolites in feces is probably a consequence of SREMs formed in enterocytes being effluxed back into the lumen of the small intestine (Actis-Goretta et al., 2013) and passing to the colon where they are converted to glucuronide and sulfate 5C-RFMs.

4. Concluding remarks

A selective, sensitive, and precise UHPLC-HRMS method was validated to identify and quantify 5C-RFMs and phenolic acid and aromatic catabolites in urine, plasma and feces of rats. The UHPLC-HRMS method allowed the simultaneous determination of 3 parent flavan-3-ols, 4 5C-RFMs as well as 27 phenolic and aromatic acid catabolites in

Table 5
Quantification of flavan-3-ols, 5 carbon side chain ring fission metabolites (5C-RFMs) phenyl-γ-valerolactones and phenolic and aromatic acids in urine, plasma and feces collected 0–24 h after feeding rats a red wine proanthocyanidin extract. Results are expressed as mean values ± SE (n = 3).

Metabolites	Urine nmol	Plasma C_{max} (nmol L^{-1})	T_{max} (h)	Feces nmol
Flavan-3-ols				
(−)-Epicatechin	0.12 ± 0.01	14.3 ± 0.4	1	0.01 ± 0.01
Procyanidin B1	0.3 ± 0.2	4.2 ± 0.1	1	–
Procyanidin B2	0.2 ± 0.2	3.6 ± 0.1	1	–
Total Flavan-3-ol	**0.6 ± 0.4 (*0.04%*)**[a]			**0.01 ± 0.01**
5C-RFMs				
5-(Dihydroxyphenyl)-γ-valerolactone derivatives				
5-(3′,4′-Dihydroxyphenyl)- γ-valerolactone	–	–	–	1.4 ± 0.3
5-(Hydroxphenyl)-γ-valerolactone derivatives				
5-(3′-Hydroxyphenyl)-γ-valerolactone	–	–	–	0.12 ± 0.02
5-(Phenyl)-γ-valerolactone-3′-sulfate	33.7 ± 0.5	70 ± 3	8	0.04 ± 0.01
5-(Phenyl)-γ-valerolactone-3′-*O*-glucuronide	11.1 ± 0.1	45 ± 2	8	–
Total 5C-RFMs	**44.8 ± 0.6 (*0.3%*)**[b]			**0.16 ± 0.03 (*0.001%*)**[b]
Phenolic And Aromatic Acid Derivatives				
Phenylpropanoid acid derivatives				
3′-Hydroxycinnamic acid	37 ± 3	–	–	–
Coumaric acid-3′-*O*-glucuronide	13.1 ± 0.1	–	–	0.9 ± 0.1
Caffeic acid-3′-*O*-glucuronide	8.2 ± 0.0	–	–	–
Ferulic acid	12 ± 2	–	–	63 ± 4
Ferulic acid-4′-sulfate	503 ± 57	310 ± 12	24	78 ± 4
Ferulic acid-4′-*O*-glucuronide	38 ± 0.2	–	–	1.6 ± 0.1
Isoferulic acid-3′-*O*-glucuronide	5.5 ± 0.1	–	–	–
Total phenylpropanoid acid derivatives	**617 ± 62**			**143 ± 8**
Phenylpropionic acid derivatives				
3-(3′-Methoxy-4′-hydroxyphenyl)propionic acid	97 ± 4	–	–	16.4 ± 0.2
3-(3′-Methoxyphenyl)propionic acid-4′-*O*-sulfate	106 ± 20	278 ± 16	24	12 ± 2
3-(3′-Methoxyphenyl)propionic acid-4′-*O*-glucuronide	25 ± 0.6	–	–	0.8 ± 0.3
3-(3′-Hydroxy-4′-methoxyphenyl)propionic acid	123 ± 2	–	–	35 ± 3
3-(3′-Hydroxyphenyl)propionic acid-4′-*O*-glucuronide	1.3 ± 0.1	–	–	9.5 ± 0.9
3-(4′-Hydroxyphenyl)propionic acid-3′-*O*-glucuronide	2.2 ± 0.2	–	–	4 ± 2
3-(3′-Hydroxyphenyl)propionic acid	150 ± 11	2568 ± 76	24	–
3-(4′-Hydroxyphenyl)propionic acid	13.8 ± 0.1	595 ± 54	6	–
3-(Phenyl)propionic acid-3′-sulfate	595 ± 8	1405 ± 101	24	–
3-(Phenyl)propionic acid-3′-*O*-glucuronide	91 ± 0.4	130 ± 13	24	–
3-Phenyllactic acid	61 ± 1	283 ± 11	6	–
Total phenylpropionic acid derivatives	**1265 ± 47**			**78 ± 8**
Phenylacetic acid derivatives				
3′-Hydroxy-4′-methoxyphenylacetic acid	43 ± 4	–	–	40 ± 13
3′-Methoxy-4′-hydroxyphenylacetic acid	16 ± 3	–	–	44 ± 8
3′-Hydroxyphenylacetic acid	37 ± 1	42 ± 3	1	228 ± 40
4′-Hydroxyphenylacetic acid	178 ± 55	683 ± 41	1	77 ± 58
Total phenylacetic acid derivatives	**274 ± 63**			**389 ± 119**
Benzoic acid derivatives				
4-Hydroxybenzoic acid	27.5 ± 0.4	2660 ± 87	2	52 ± 10
3-Hydroxybenzoic acid	nd	129 ± 11	4	–
Total benzoic acid derivatives	**27.5 ± 0.4**			**52 ± 10**
Hydroxycarboxylic acid derivatives				
4′-Hydroxymandelic acid	26 ± 1	205 ± 11	4	14 ± 5
Total hydroxycarboxylic acid derivatives	**26 ± 1**			**14 ± 5**
Benzenetriol derivatives				
1,3,5-Trihydroxybenzene (Phloroglucinol)	2.7 ± 0.4	–	–	–
Total benzenetriol derivatives	**2.7 ± 0.4**			**–**
Benzoylglycine derivatives				
4′-Hydroxyhippuric acid	80 ± 2	68 ± 5	4	7 ± 2
Hippuric acid	275 ± 55	433,478 ± 2180	24	–
Total benzoylglycine derivatives	**355 ± 57**			**7 ± 2**
Total phenolic and aromatic acid derivatives	**2567 ± 230 (*17.3*%)**			**683 ± 152 (*4.6*%)**

[a] Figure in italicised parentheses represents recovery as a percentage of intake of flavan-3-ol monomers (1.5 μmol).
[b] Figure in italicised parentheses represents recovery as a percentage of monomers and proanthocyanidin oligomers and polymers (14.8 μmol).

rat urine, plasma and feces samples collected 0–24 h after gavage of a proanthocyanidin-rich red wine extract. The total urinary excretion of 5C-RFMs was equivalent to 0.3% of intake and that of phenolic acid and aromatic catabolites derived from the colon, 17.3% of the ingested proanthocyanidin extract. Overall the study provided a detailed evaluation of the absorption, metabolism and catabolism of the red wine proanthocyanidin extract in rats and highlighted the important role of the colonic microbiota in the bioavailability of these compounds.

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Notes

The authors declare no competing financial interest

Fundings

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgements

GP-C is supported by a postdoctoral research contract “Juan de la Cierva-Incorporación” funded by the Spanish Ministry of Economy and Competitiveness (FJCI-2015-26433). JLO was granted a research contract funded at Andalusian Institute of Agricultural and Fisheries Research and Training (IFAPA), inside the National Youth Guarantee System funded through the European Social Fund (ESF) and the Youth Employment Initiative (YEI). IAL was supported by a postdoctoral research contract “Juan de la Cierva-Formación” funded by the Spanish Ministry of Economy and Competitiveness (FJCI-2014-20689).

References

Actis-Goretta, L., Lévèques, A., Rein, M., Teml, A., Schäfer, C., Hofman, U., ... Williamson, G. (2013). Intestinal absorption, metabolism, and excretion of (−)-epicatechin in healthy humans assessed by using an intestinal perfusion technique. *American Journal of Clinical Nutrition, 98*, 924–933.

AOAC (1993). Peer Verified methods Program. *Manual of policies and procedures. Arlngton, Virginia, USA*.

Baba, S., Osakabe, N., Natsume, M., & Terao, J. (2002). Absorption and urinary excretion of procyanidin B2 [epicatechin-(4β-8)-epicatechin] in rats. *Free Radical Biology and Medicine, 33*, 142–148.

Bindon, K. A., & Smith, P. A. (2013). Comparison of the affinity and selectivity of insoluble fibres and commercial proteins for wine proanthocyanidins. *Food Chemistry, 136*, 917–928.

Borges, G., van der Hooft, J. J., & Crozier, A. (2016). A comprehensive evaluation of the [2-^{14}C](-)-epicatechin metabolome in rats. *Free Radical Biology and Medicine, 99*, 128–138.

Borges, G., Ottaviani, J. I., van der Hooft, J. J. J., Schroeter, H., & Crozier, A. (2017). Absorption, metabolism, distribution and excretion of (−)-epicatechin: A review of recent findings. *Molecular Aspects of Medicine*. http://dx.doi.org/10.1016/j.mam.2017.11.002 E-pub, ahead of print.

Brindani, N., Mena, P., Calani, L., Benzie, I., Choi, S. W., Brighenti, F., ... Del Rio, D. (2017). Synthetic and analytical strategies for the quantification of phenyl-γ-valerolactone conjugated metabolites in human urine. *Molecular Nutrition and Food Research, 61* (doi: 10.1002/mnfr.20170007).

Crozier, A., Jaganath, I. B., & Clifford, M. N. (2006). Phenols, polyphenols and tannins: an overview. In A. Crozier, M. N. Clifford, & H. Ashihara (Eds.). *Plant secondary metabolites: Occurrence, structure and role in the human diet* (pp. 1–24). Oxford: Blackwell Publishing.

Curti, C., Brindani, N., Battistini, L., Sartori, A., Pelosi, G., Mena, P., ... Del Rio, D. (2015). Catalytic, enantioselective vinylogous mukaiyama aldol reaction of furan-based dienoxy silanes: a chemodivergent approach to γ-valerolactone flavan-3-ol metabolites and δ-lactone analogues. *Advances in Synthesis and Catalysis, 357*, 4082–4092.

Del Rio, D., Calani, L., Cordero, C., Salvatore, S., Pellegrini, N., & Brighenti, F. (2010). Bioavailability and catabolism of green tea flavan-3-ols in humans. *Nutrition, 26*, 1110–1116.

Del Rio, D., Rodríguez-Mateos, A. M., Spencer, J. P. E., Tognolini, M., Borges, G., & Crozier, A. (2013). Dietary (poly)phenolics in Human Health: structures, bioavailability and evidence of protective effects against chronic diseases. *Antioxidant Redox Signal, 18*, 1818–1892.

Ding, Y., Peng, M., Zhang, T., Tao, J. S., Cai, Z. Z., & Zhang, Y. (2013). Quantification of conjugated metabolites of drugs in biological matrices after the hydrolysis with β-glucronidase and sulfatase: a review of bio-analytical methods. *Biomedicine and Chromatography, 27*, 1280–1295.

Feliciano, R. P., Krueger, C. G., & Reed, J. D. (2015). Methods to determine effects of cranberry proanthocyanidins on extraintestinal infections: relevance for urinary tract health. *Molecular Nutrition and Food Research, 59*, 1292–1306.

Feliciano, R. P., Pritzel, S., Heiss, C., & Rodríguez-Mateos, A. M. (2015). Flavonoid intake and cardiovascular disease risk. *Current Opinion in Food Science, 2*, 92–99.

Feliciano, R. P., Mecha, E., Bronze, M. R., & Rodriguez-Mateos, A. M. (2016). Development and validation of a high-throughput micro solid-phase extraction method coupled with ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry for rapid identification and quantification of phenolic metabolites in human plasma and urine. *Journal of Chromatography A, 1464*, 21–31.

Ferrars, R. M., Czank, C., Saha, S., Needs, P. W., Zhang, Q., Raheem, K. S., ... Kay, C. (2014). Methods for isolating, identifying, and quantifying anthocyanin metabolites in clinical samples. *Analytical Chemistry, 86*, 10052–10058.

Gasperotti, M., Masuero, D., Guella, G., Mattivi, F., & Vrhovsek, U. (2014). Development of a targeted method for twenty-three metabolites related to polyphenol gut microbial metabolism in biological samples using SPE and UHPLC-ESI-MS/MS. *Talanta, 128*, 221–230.

Goodrich, K. M., & Neilson, A. P. (2014). Simultaneous UPLC-MS/MS analysis of native catechins and procyanidins and their microbial metabolites in intestinal contents and tissues of male Wistar Furth inbred rats. *Journal of Chromatography B, 958*, 63–74.

Granato, D., Santos, J. S., Maciel, L. G., & Nunes, D. S. (2016). Chemical perspective and criticism on selected analytical methods used to estimate the total content of phenolic compounds in food matrices. *Trends in Analytical Chemistry, 80*, 266–279.

Llorach, R., Urpi-Sarda, M., Tulipani, S., García-Aloy, M., Monagas, M., & Andres-Lacueva, C. (2013). Metabolomic fingerprinting in patients at high risk of cardiovascular disease by cocoa intervention. *Molecular Nutrition and Food Research, 57*, 962–973.

Ludwig, I. A., Mena, P., Calani, L., Borges, G., Pereira-Caro, G., Bresciani, L., ... Crozier, A. (2015). New insights into the bioavailability of red raspberry anthocyanins and ellagitannins. *Free Radical Biology and Medicine, 89*, 758–769.

Magnusson, B. & Örnemark, U. (2014). Eurachem Guide: The Fitness for Purpose of Analytical Methods- A Laboratory Gide to Method Validation and Related Topics. (2nd Ed.). Available from http://www.eurachem.org.

Ottaviani, J. I., Borges, G., Momma, T. Y., Spencer, J. P., Keen, C. L., Crozier, A., & Schroeter, H. (2016). The metabolome of [2-(14)C](-)-epicatechin in humans: implications for the assessment of efficacy, safety, and mechanism of action of polyphenolic bioactives. *Scientific Report, 6*, 29034.

Robbins, R. J., Leonczak, J., Johnson, J. C., Li, J., Kwik-Uribe, C., Prior, R. L., & Gu, L. (2009). Method performance and multi-laboratory assessment of a normal phase high pressure liquid chromatography-fluorescence detection method for the quantification of flavanols and procyanidins in cocoa and chocolate containing samples. *Journal of Chromatography A, 1216*, 4831–4840.

Rodríguez-Mateos, A. M., Vauzour, D., Krueger, G., Shanmuganayagam, D., Reed, J., Calani, L., ... Crozier, A. (2014). Bioavailability, bioactivity and impact on health of dietary flavonoids and related compounds: an update. *Achieves in Toxicology, 88*, 1803–1853.

Saenger, T., Hubner, F., & Humpf, H. U. (2017). Short-term biomarkers of apple consumption. *Molecular Nutrition and Food Research, 61* (doi: 10.1002/mnfr.201600629).

Saha, S., Hollands, W., Needs, P. W., Ostertag, L. M., de Roos, B., Duthie, G. G., & Kroon, P. A. (2012). Huma O-sulfated metabolites of (−)-epicatechin and methyl-(−)-epicatechin are poor substrates for commercial aryl-sulfatases: implications for studies concerned with quantifying epicatechin bioavailability. *Pharmacology Research, 65*, 592–602.

Sasot, G., Martínez-Huélamo, M., Vallverdú-Queralt, A., Mercader-Marti, M., Estruc, R., & Lamuela-Raventós, R. M. (2017). Identification of phenolic metabolites in human urine after the intake of a functional food made from grape extract by high resolution LTQ-Orbitrap-MS approach. *Food Research International, 100*, 435–444.

Serra, A., Maciá, A., Romero, M. P., Salvadó, M. J., Bustos, M., Fernández-Larrea, J., & Motilva, M. J. (2009). Determination of procyanidins and their metabolites in plasma samples by improved liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B, 877*, 1169–1176.

Serra, A., Maciá, A., Romero, M. P., Piñol, C., & Motilva, M. J. (2011). Rapid methods to determine procyanidins, anthocyanins, theobromine and caffeine in rat tissues by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B, 879*, 1519–1528.

Shoji, T., Masumoto, S., Moriichi, N., Akiyama, H., Kanda, T., Ohtake, Y., & Goda, Y. (2006). Apple procyanidin oligomers absorption in rats after oral administration: analysis of procyanidins in plasma using the porter method and high-performance liquid chromatography-tandem mass spectrometry. *Journal of Agricultural and Food Chemistry, 54*, 884–892.

Tsang, C., Auger, C., Mullen, W., Bornet, A., Rouanet, J. M., Crozier, A., & Teissedre, P. L. (2005). The absorption, metabolism and excretion of flavan-3-ol and procyanidins following the ingestion of a grape seed extract by rats. *British Journal of Nutrition, 94*, 170–181.

Ulaszewska, M. M., Trost, K., Stanstrup, J., Tuohy, K. M., Franceschi, P., Chong, M. F. F., ... Mattivi, F. (2016). Urinary metabolimic profiling to identify biomarkers of a flavonoid-rich and flavonoid-poor fruits and vegetables diet in adults: the FLAVUR trial. *Metabolomics, 12*, 32.

Urpi-Sarda, M., Monagas, M., Kham, N., Lamuela-Raventos, R. M., Santos-Buelga, C., Sacanella, E., ... Andres-Lacueva, C. (2009). Epicatechin, procyanidins, and phenolic

microbial metabolites after cocoa intake in humans and rats. *Analytical and Bioanalytical Chemistry, 394*, 1545–1556.
Urpi-Sarda, M., Monagas, M., Khan, N., Llorach, R., Lamuela-Raventos, R. M., Jauregui, O., ... Andres-Lacueva, C. (2009). Targeted metabolomic profiling of phenolics in urine and plasma after regular consumption of cocoa by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A, 1216*, 7258–7267.
Wiese, S., Esatbeyoglu, T., Winterhalter, P., Kruse, H. P., Winkler, S., Bub, A., & Kulling, S. E. (2015). Comparative biokinetics and metabolism of pure monomeric, dimeric, and polymeric flavan-3-ols: a randomized cross-over study in humans. *Molecular Nutrition and Food Research, 59*, 610–621.
Williamson, G., & Clifford, M. N. (2017). Role of the small intestine, colon and microbiota in determining the metabolic fate of polyphenols. *Biochemical Pharmacology, 139*, 24–39.
Xiao, Y., Hu, Z., Yin, Z., Zhou, Y., Liu, T., Zhou, X., & Chang, D. (2017). Profiling and distribution of metabolites of procyanidin B2 in mice by UPLC-DAD-ESI-IT-TOF-MSn technique. *Frontiers in Pharmacology, 8*, 231.
Zanotti, I., Dall'Asta, M., Mena, P., Mele, L., Bruni, R., Ray, S., & Del Rio, D. (2015). Atheroprotective effects of (poly)phenols: a focus on cell cholesterol metabolism. *Food & Function, 6*, 13–31.
Zhang, H., & Tsao, R. (2016). Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects. *Current Opinion on Food Science, 8*, 33–42.
Zhang, Y., Huo, M., Zhou, J., & Xie, S. (2010). PKSolver: an add-in program for pharmacokinetic and pharmacodynamics data analysis in Microsoft Excel. *Computational Methods and Programs in Biomedicine, 99*, 306–314.

DISCUSIÓN GENERAL

Discusión general

En esta sección se presenta una discusión general de los resultados obtenidos en los capítulos que conforman la Tesis Doctoral. Tal y como indican los objetivos de esta tesis, los trabajos se han centrado en el desarrollo de metodologías analíticas específicas para la caracterización y cuantificación de compuestos con actividad biológica en productos vegetales, bebidas fermentadas y muestras biológicas. Estos nuevos métodos se han aplicado a la resolución de problemas agrupados en dos grandes bloques temáticos, Seguridad alimentaria (**Objetivos 1, 2 y 3**) y Calidad Alimentaria (**Objetivos 4 y 5**).

Seguridad Alimentaria

Como ya se ha visto, la seguridad alimentaria tiene como principal objetivo la protección de la salud de las personas en relación con los alimentos ingeridos. Las aminas biógenas son un grupo de biomoléculas con potencial efecto adverso a los consumidores. En relación a estos compuestos, el Panel de Riesgo Biológico de la EFSA, elaboró en 2011 una opinión científica sobre la formación de aminas biógenas en alimentos fermentados, en la que se indica la importancia del control de estos compuestos en los alimentos y se recomienda la validación de métodos analíticos para las diferentes matrices donde estén presentes.

En relación al **Objetivo 1**, se realizó una revisión bibliográfica sobre los métodos analíticos para la determinación de aminas biógenas. Entre las técnicas analíticas más empleadas se encuentra la cromatografía líquida, la cromatografía de gases y la electroforesis capilar (Peña-Gallego et al., 2012). De todas ellas, la cromatografía líquida fue la más utilizada, ya que es una técnica analítica con gran versatilidad, permite obtener el perfil de aminas biógenas y está presente comúnmente en los laboratorios analíticos. Los métodos de cromatografía líquida que emplean una columna tipo C18 en fase reversa son las más comunes debido a su alta resolución y sensibilidad (Hernández-Orte et al., 2006).

Previamente al análisis cromatográfico, es recomendable una preparación de las muestras para disminuir las interferencias de otros compuestos (efecto matriz) o para concentrar los analitos de interés (Sentellas et al., 2016). De esta forma, respecto a la extracción de las muestras de bebidas fermentadas, aunque algunos métodos usan una extracción líquida-líquida o extracción en fase sólida (SPE), la tendencia actual va encaminada al empleo de métodos como las micro extracciones en fase líquida (LPME) o fase sólida (SPME), los cuales son más rápidos, usan menos volumen de solventes orgánicos y obtienen mejores valores de recuperación (Basheer et al., 2011; Almeida et al., 2012).

Con el objeto de mejorar la sensibilidad y la separación en fase reversa, es común el uso de reactivos derivatizantes. En general, no existe un agente derivatizante universal, ya que todos los reactivos usados tienen ventajas e inconvenientes. Así, los reactivos presentan diferentes valores de sensibilidad, especificidad, número de analitos, tiempo de reacción, estabilidad… (Peña-Gallego et al., 2012). En el periodo evaluado (2010-2016), el cloruro de dansilo fue el reactivo derivatizante más empleado, seguido de *o*-ftalaldehído, cloruro de benzoilo, 6-aminoquinolil-N-hidroxisuccinimidil carbamato y etoximetilenmalonato de dietilo.

En cuanto a la detección, los detectores más usados son de ultravioleta o fluorescencia, dependiendo del tipo de reactivo derivatizante usado. Recientemente, el uso de espectrómetros de masas ha ido aumentando, sobre todo llevando a cabo el análisis de estos compuestos en modo de ionización positiva. Generalmente, los métodos que usan los detectores de fluorescencia muestran mejores valores de sensibilidad frente a los de ultravioleta, y no se observó una diferencia significativa en la sensibilidad de los métodos evaluados que emplean detectores de masas, por lo que no se justifica el coste de estos últimos.

Las principales aminas biógenas detectadas en las bebidas fermentadas son la histamina, la tiramina, la putrescina y la cadaverina, producidas principalmente por las bacterias lácticas. Los objetivos principales del estudio de las aminas biógenas en las bebidas fermentadas se pueden englobar en tres líneas: Seguridad Alimentaria, Control del proceso de producción o Estudios microbiológicos de los alimentos.

Los retos actuales a lo que se enfrenta la investigación de las aminas biógenas están relacionados con la reducción del tiempo de análisis, la reducción de la concentración de reactivo y el aumento de la sensibilidad. De este modo, en el contexto de una futura regulación de las aminas biógenas en productos fermentados, serán necesarios la existencia y utilización de unos métodos analíticos rápidos y robustos.

En general, la determinación de concentraciones de aminas biógenas menores a mg/L no es necesaria en relación a la seguridad alimentaria. De este modo, la legislación actual en productos de pesca así como las recomendaciones establecidas para la histamina en vino y cerveza tiene unos niveles del orden de mg/L. Por ello, los métodos de cromatografía acoplados a detectores de UV o fluorescencia son adecuados para determinar las aminas biógenas en el laboratorio. Sin embargo, en relación al control de calidad y la investigación, son necesarios métodos más sensibles y una identificación más precisa para detectar ligeros cambios en el perfil de aminas biógenas. De esta forma, los detectores de masas son los más adecuados para la detección de metabolitos a bajas concentraciones, ya que son más sensibles y aportan información estructural específica.

El **Objetivo 2** se encuentra enmarcado dentro del proyecto: "Fermentación selectiva para la producción de una nueva bebida a partir de fresa no apta para comercialización" (AGL2010-22152-C03-01/02/03). Más concretamente, dentro del subproyecto "Evaluación de la calidad y seguridad de una nueva bebida obtenida a partir de fresa no apta para comercialización". Para el desarrollo del proyecto fue necesario conocer la influencia que los procesos fermentativos ejercen sobre la composición química de esta nueva bebida y la seguridad de su consumo. Así, el propósito fue comprobar que las bacterias elegidas para llevar a cabo la fermentación, usaran la glucosa de modo selectivo manteniendo la fructosa inalterada y no produjeran aminas biógenas. Para ello, fue preciso disponer de métodos validados para la determinación de estos compuestos no deseados en las bebidas de fresa. Debido a la versatilidad del método empleado, de manera simultánea se pudo determinar los aminoácidos durante el proceso fermentativo ofreciendo un seguimiento adicional en la transformación de esta bebida.

Debido a que no todos los reactivos derivatizantes pueden ser empleados para determinar aminas biógenas y aminoácidos de forma simultánea, fue seleccionado el etoximetilenmalonato de dietilo (DEEMM) como reactivo derivatizante precolumna, ya que puede reaccionar con los grupos aminos primarios y secundarios dando lugar a derivados detectables en la región UV-Vis. De este modo, un método cromatográfico desarrollado originalmente por Gómez-Alonso et al. (2007) para determinar aminoácidos, aminas biógenas y amonio en vinos y cervezas fue adaptado para la determinación de estos compuestos nitrogenados en puré de fresa y su fermentado glucónico. Este método ya ha sido usado en gran diversidad de muestras, tales como vino, cerveza, vinagre, miel, queso (Jia et al., 2011; Martínez-Pinilla et al., 2013; Redruello et al., 2013; Chinnici et al., 2016). Para mejorar la resolución de los picos en los cromatogramas de las fresas y sus productos fermentados, se realizaron ligeras modificaciones en el gradiente y la temperatura (45 ºC), manteniendo el resto de parámetros. En total se determinaron 31 compuestos, incluidos 22 aminoácidos, 8 aminas biógenas e ion amonio. El nuevo método se validó en términos de la respuesta de linealidad, la sensibilidad [límites de detección (LD) y cuantificación (LQ)] y la precisión (repetibilidad y precisión intermedia) de acuerdo con los criterios establecidos por la guía elaborada por la "*Association of Analytical Communities"* (AOAC) (AOAC, 1993). El coeficiente de determinación (R^2) fue superior a 0.99 para todas las rectas de calibración, mostrando una buena relación linear entre la concentración de los estándares y la respuesta del detector. Los LD variaron de 0.05 a 0.3 mg/L para todos los analitos, mientras que los LQ fueron entre 0.20 a 0.95 mg/L, siendo estos similares a los obtenidos por Gómez-Alonso et al. (2007). En cuanto a la precisión, tanto la repetibilidad como la precisión intermedia tuvieron una desviación estándar relativa (RSD) inferior al 15%. Debido a la alta concentración de asparagina y su proximidad a serina, el solapamiento de los picos no se pudo evitar y ambos se cuantificaron como asparagina-serina.

Una vez validado, el método fue aplicado para determinar aminas biógenas, aminoácidos y amonio en puré de fresa y fermentados glucónicos obtenidos mediante cultivos superficiales por tres cepas de bacterias acéticas: *Acetobacter malorum, Gluconobacter oxydans* y *Gluconobacter japonicus* para ver los cambios producidos en el perfil de estos compuestos durante la fermentación.

Las aminas biógenas no fueron detectadas en el sustrato de fresa, posiblemente porque estos compuestos se encuentran en bajas concentraciones de forma endógena (Önal, 2007; Cipolla et al., 2010). Del mismo modo, los fermentados glucónicos de fresa tampoco presentaron concentraciones detectables de aminas biógenas. Estos resultados están de acuerdo con Landete et al. (2007), quienes observaron que durante los procesos fermentativos las principales productoras de aminas biógenas son las bacterias lácticas a diferencia de las bacterias acéticas. Sin embargo, la información en relación a la producción de aminas biógenas por las bacterias del género *Gluconobacter* era escasa. Por todo ello se pudo concluir que la elaboración de bebidas con estos fermentados fue segura para el consumo humano.

Por otro lado, los aminoácidos mayoritarios en las muestras de fresa fueron asparagina-serina, alanina y glutamina, estando de acuerdo con lo observado en estudios previos (Pérez et al., 1992, Moing et al., 2001). Tras las fermentaciones, los cambios en el perfil de aminoácidos fueron similares para las tres cepas empleadas. Así, los principales aminoácidos que disminuyeron su concentración fueron glutamina, alanina y triptófano, posiblemente utilizados como fuente de nitrógeno por las bacterias acéticas. Por el contrario, los aminoácidos que incrementaron su concentración fueron arginina, GABA y prolina. En todas las muestras fermentadas, la asparagina-serina fue el aminoácido más abundante seguido de la arginina, el ácido glutámico, la prolina y el ion amonio.

Además, se realizaron dos tipos de análisis discriminante lineal (LDA), usando los métodos de selección de variable tipo estándar y *forward stepwise*, para evaluar si el perfil de aminoácidos y el ion amonio eran lo suficientemente diferentes como para distinguir las muestras analizadas en este estudio en función del sustrato y la cepa bacteriana. Se observó que ambos análisis fueron capaces de separar el sustrato de los productos de ácido glucónico, así como las cepas entre ellas, a pesar de tener un perfil similar de aminoácidos.

En relación al proyecto, uno de sus objetivos fue la selección de una cepa de bacteria acéticas que transformará la glucosa en ácido glucónico y manteniendo la fructosa original del zumo de fresa. De las 3 cepas evaluadas, *G. japonicus* fue la cepa seleccionada para la elaboración de la bebida ya que mostró una mayor actividad durante la fermentación, consumiendo la glucosa más rápido.

De esta forma, se llevaron a cabo varias fermentaciones glucónicas con cultivo sumergido utilizando la cepa seleccionada. Este tipo de fermentación proporcionó una alternativa mucho más rápida que la fermentación con cultivo superficial y más interesante para aplicarla a escala industrial. Así, la transformación de glucosa en ácido glucónico tuvo una duración de 8 horas frente a los 10 días de la fermentación con cultivo superficial. Además, de la misma forma que en el caso anterior, *G. japonicus* mostró ser bastante selectiva con el uso de glucosa, manteniendo constante la fructosa procedente del sustrato en el producto final. La fermentación con cultivo sumergido no formó aminas biógenas, al igual que en el estudio previo. En este caso, no se observaron cambios entre sustrato y el producto final en el perfil de aminoácidos ni en el contenido total de nitrógeno. Así el contenido total de nitrógeno al inicio de la fermentación fue de 19.91 ± 0.93 mM, mientras que el final fue de 20.43 ± 1.21 mM. Las diferencias entre los dos tipos de fermentaciones pueden ser debidas a que la fermentación con cultivo superficial presentó un metabolismo activo y de crecimiento bacteriano, mientras que la fermentación con cultivo sumergido tuvo un metabolismo orientado a una actividad catalítica. Además, también podría deberse a la notable diferencia en la duración de la fermentación.

A pesar de las pocas diferencias en el perfil de aminoácidos e ion amonio, el análisis PCA fue capaz de agrupar las muestras iniciales y finales usando como variables lisina, fenilalanina, ácido glutámico, isoleucina, histidina, ácido aspártico, leucina, alanina e ion amonio.

Por todo ello, se puede concluir que el fermentado glucónico es seguro para el consumo humano en relación a la cantidad de aminas biógenas. Por otro lado, la fermentación glucónica con cultivo sumergido mantiene la composición de aminoácidos en el producto final, además de conservar los compuestos bioactivos (Álvarez-Fernández et al., 2014; Hornedo-Ortega et al., 2016).

El **Objetivo 3** se centró en la puesta a punto y validación de un método sensible para determinar aminas biógenas en productos finales (vinagres) o en la conservación de alimentos (vinos abiertos). Aunque en el capítulo anterior se valida y aplica un método para determinar aminas biógenas en productos fermentados, es importante disponer de métodos que sean más específicos y sensibles para poder determinar concentraciones más pequeñas y evitar la interferencias de otros compuestos, como los aminoácidos. En este caso se seleccionó el 6-aminoquinolil-N-hidroxisuccinimidil carbamato (AQC) como reactivo derivatizante precolumna, ya que es un reactivo más estable, robusto y sensible que el DEEMM. Así, el AQC reacciona con los grupos aminos, formando derivados que incrementan su sensibilidad a detectores de fluorescencia frente al DEEMM, el cual mejora la detección por ultravioleta (Peña-Gallego et al., 2012).

De este modo, un método cromatográfico desarrollado originalmente por Peña-Gallego et al. (2009) para determinar aminas biógenas en vinos usando AQC como agente derivatizante fue validado para la determinación de estos compuestos nitrogenados en vinagres. En orden de evitar interferencias de otros compuestos, principalmente aminoácidos, se utilizó una extracción en fase sólida (SPE) con un cartucho de resinas de modo mixto (Oasis MCX 1cc) antes del análisis cromatográfico.

En relación al método analítico, este fue validado para la determinación de aminas biógenas en vinagres, ampliando el número de analitos del método original de 4 a 9 (histamina, tiramina, putrescina, cadaverina, agmatina, espermidina, espermina, metilamina y feniletilamina). El método fue validado exitosamente en términos de selectividad, linealidad, sensibilidad (LD y LQ), precisión (repetibilidad y precisión intermedia) y recuperación de acuerdo con los criterios establecidos por AOAC (AOAC, 1993). La selectividad fue evaluada como la resolución entre los picos adyacentes (R), siendo esta mayor de 1,5 para todas las aminas biógenas. El R^2 osciló entre 0.991 y 0.999 para todas las rectas de calibración, mostrando una buena relación linear entre la concentración de los estándares y la respuesta del detector. Los LD variaron de 7 a 26 μg/L para todos los analitos, mientras que los LQ fueron entre 17 a 68 μg/L, siendo estos similares a los obtenidos por métodos que emplean AQC (Hernández-Orte et al. 2006; Peña-Gallego et al., 2009) y más sensibles que los métodos con DEEMM (Gómez-Alonso et al., 2007). En cuanto a la precisión, tanto la repetibilidad (precisión intradía) como la precisión intermedia (precisión interdía) tuvieron una desviación estándar relativa (RSD) inferior al 15%. La exactitud se evaluó a dos concentraciones, obteniendo una tasa de recuperación para la mayoría de los analitos en un rango de 80 al 110%.

El método validado fue empleado para determinar aminas biógenas en diferentes tipos de vinagres, entre los que se encuentran 6 tipos de vino tinto, 4 de vino blanco, 3 de manzana y 3 balsámicos. Además se evaluaron 10 vinagres de vino de Jerez, divididos en 3 vinagres de Jerez, 3 vinagres de Jerez Reserva, 3 vinagres de Jerez Gran Reserva y 1 Pedro Ximénez.

El contenido total de aminas biógenas en vinagres osciló entre 23.35 y 1445.2 μg/L. La metilamina y la feniletilamina no fueron detectadas en ningún vinagre. Por el contrario, la putrescina seguida de la histamina, fueron las aminas biógenas que mostraron las concentraciones más altas en algunos vinagres, alcanzando valores de hasta 525 y 309 μg/L, respectivamente. Los vinagres con mayor concentración de aminas fueron los balsámicos, el Pedro Ximénez y algunos de los vinagres de vino tinto. Por el contrario, los vinagres de Jerez y vinagres de manzana tenían menor concentración de estos compuestos.

Por otro lado, se llevó a cabo un análisis de componentes principales (PCA) para evaluar si el perfil de aminas biógenas era suficiente para diferenciar entre los distintos tipos de vinagres analizados en el estudio. De esta forma, el primer factor permitió separar los vinagres con menor concentración de aminas biógenas (vinagres de manzana, Jerez y vino blanco) y los vinagres con mayor concentración (vinagre balsámico y Pedro Ximenez). El segundo factor separó las muestras según la concentración de espermina. En este caso, las muestras de vinagre de vino blanco y algunos vinos tintos, se separaron del resto. Los vinagres de vino tinto se encontraron distribuidos de forma heterogénea debido a que el perfil de aminas biógenas en las muestras fue muy variable.

Por lo general, las cantidades de aminas biógenas cuantificadas en los vinagres analizados son inferiores a las encontradas en otros productos fermentados, como el vino o el queso, y por tanto no alcanzan niveles que provoquen efectos perjudiciales para el consumo humano.

Por otro parte, los estudios presentes en la bibliografía en relación al perfil de aminas biógenas en los vinos finales están centrados en la conservación en botellas cerradas. Sin embargo, los vinos suelen mantenerse en botellas abiertas en el sector de la restauración, por lo que es importante controlar la evolución de estos compuestos a lo largo del tiempo. Para este estudio se seleccionaron tres tipos de vinos: un vino tinto de calidad media, un vino tinto de alta calidad y un vino blanco joven. Una vez abiertos, las botellas se conservaron en diferentes condiciones de almacenamiento (temperatura y tipo de tapón). Así, las botellas se mantuvieron a temperatura ambiente (25 ºC) o fría (4 ºC). Con respecto al tipo de tapón, las botellas fueron tapadas con un tapón corcho o con un tapón con un sistema de bomba de vacío para extraer el aire de la botella (Vacu Vin®).

La concentración media de aminas biógenas en las botellas recién abiertas 37.7 mg/L, 21.2 mg/L y 5.08 mg/L para los vinos tintos de calidad media, de alta calidad y vino blanco, respectivamente. Los dos vinos tintos mostraron mayores cantidades de histamina y putrescina, mientras que el vino blanco tenía una concentración similar de tiramina, putrescina e histamina. Cadaverina presentó una concentración similar para todas las muestras, oscilando de 0.25 a 0.42 mg/L. Por otro lado, metilamina y espermina no se detectaron en ninguna muestra.

Durante el almacenamiento de las botellas abiertas de vino en las diferentes condiciones se observaron ligeros cambios en el perfil de aminas biógenas. En el caso del vino tinto de calidad media, la concentración total de aminas biógenas tuvo una tendencia a disminuir a lo largo del tiempo en todas las condiciones de almacenamiento, principalmente histamina y cadaverina disminuyeron significativamente. Sin embargo, otras aminas minoritarias aumentaron, tales como feniletilamina y espermidina. Respecto al vino tinto de calidad alta, si se observaron algunas diferencias entre las diferentes condiciones de almacenamiento. Así, las muestras no

almacenadas al vacío mostraron una tendencia a disminuir con el tiempo, a diferencia de los mantenidos al vacío donde no se observó cambios en las concentraciones totales. Estas diferencias se deben principalmente a la tiramina, ya que esta amina biógena disminuyó significativamente desde el cuarto día de almacenamiento en aquellos vinos mantenidos en condiciones de vacío. Al igual que en el caso anterior, la histamina también disminuyó a lo largo del tiempo en todas las condiciones. En el caso del vino blanco, se observó un aumento significativo en las concentraciones de histamina desde el cuarto día en todas las condiciones, a diferencia de los vinos tintos.

En general, considerando la concentración de aminas biógenas de todas las muestras, se observó una marcada correlación positiva entre histamina, tiramina, putrescina y cadaverina (r = 0.73-0.94).

Por otro lado, se llevó a cabo una PCA para evaluar si el perfil de aminas biógenas eran útiles para separar y agrupar los diferentes tipos de vino seleccionados en este estudio. El análisis separó principalmente según el tipo de vino. Además, pudo separar las muestras de vino tinto de calidad media según el tiempo de almacenamiento. Alternativamente, con el fin de explorar las diferencias entre las muestras teniendo en cuenta el tiempo de almacenamiento para cada vino, se aplicó un análisis LDA para evaluar si las concentraciones de aminas biógenas eran suficientes para diferenciar las muestras de los días 0, 4 y 10. Se llevó a cabo un análisis estándar para cada tipo de vino, obteniendo una matriz de clasificación del 100% para todos los casos. En general, los perfiles de aminas biógenas pueden separar las muestras de los tres vinos según su tiempo de almacenamiento e independientemente de las condiciones de almacenamiento, aunque las distancias fueron bastante cercanas. Especialmente el vino tinto de alta calidad mostró tener una mayor estabilidad a lo largo del tiempo.

Por todo ello, se determinó que las diferentes condiciones de almacenamiento no provocan cambios importantes en el perfil de aminas biógenas.

En general, respecto a los métodos analíticos evaluados, se observó que debido a las limitaciones del método para la determinación de forma simultanea de aminas biógenas y aminoácidos, principalmente a la sensibilidad, fue necesario el desarrollo de dos métodos diferentes según el objetivo que se persigue. Así, en el caso del método que emplea DEEMM como agente derivatizante, es necesario mejorar los límites de detección de las aminas biógenas ya que, aunque son adecuados en términos de seguridad alimentaria, son altos para objetivos de investigación, los cuales necesitan una mayor sensibilidad.

Calidad Alimentaria

En los últimos años, se aprecia una creciente demanda social por los alimentos con efectos beneficiosos sobre la salud. Así, una dieta rica en frutas y verduras está asociada a una menor prevalencia de padecer ciertas enfermedades. De esta forma, ciertos compuestos presentes en estos alimentos, denominados compuestos fenólicos, presentan una potencial actividad biológica la cual está relacionada con los efectos beneficiosos vistos en salud. No obstante, para ahondar en el conocimiento de su función biológica y en los mecanismos a través de los cuales ejercen su efecto protector es imprescindible conocer la forma en la que se absorben, metabolizan, distribuyen y excretan por el organismo, es decir, evaluar su biodisponibilidad. Además, es importante conocer las transformaciones a las que son sometidas los compuestos originales, ya que las propiedades bioactivas son ejercidas principalmente por los derivados formados.

El **Objetivo 4** está enfocado a la validación de un método analítico para determinar compuestos fenólicos y sus metabolitos en diferentes matrices biológicas tras la ingesta de un alimento rico en estos compuesto fenólicos, mediante cromatografía de líquidos acoplada a un detector de espectrometría de masas de alta resolución (HPLC-HRMS).

En primer lugar se desarrolló y validó el método cromatográfico para la determinación de derivados de metabolitos y catabolitos microbianos en muestras de orina, plasma y heces de ratas tras la ingesta de un extracto de vino rico en proantocianidinas. En este estudio, para la detección de los analitos en las muestras biológicas no fue aplicado un tratamiento con enzimas hidrolíticas, como glucuronidasas o sulfatasas, que junto al uso de un detector HRMS, nos permitió identificar metabolitos conjugados específicos, a diferencia de una gran cantidad de estudios previos (Lee et al., 2002; Chow et al., 2005; Henning et al., 2005; Donovan et al., 2006; Saha et al., 2012). Además, el método fue validado usando patrones reales, algunos de los cuales son compuestos no comerciales y fueron obtenidos por síntesis química, por lo que permitió una validación y cuantificación más precisa (Nagy et al., 2011). En total se determinaron 35 compuestos fenólicos, incluidos 3 flavanoles, 4 derivados de valerolactonas y 27 ácidos fenólicos y ácidos aromáticos en tres matrices biológicas.

El método fue validado exitosamente en términos de especificidad, linealidad, sensibilidad (LD y LQ), precisión (intradía y interdía), recuperación y efecto matriz de acuerdo con los criterios establecidos por la guía de "*Eurachem*" (Eurachem, 2014). La especificidad fue evaluada como la desviación en ppm (error de masas entre el m/z teórico y el observado), obteniendo valores menores de 5 ppm, lo cual se considera como un nivel aceptable de precisión de masa. Respecto a la linealidad, el R^2 fue mayor para todas las rectas de calibración, mostrando una buena relación linear entre la concentración de los estándares y la respuesta del detector para las tres

matrices. En general, los LD y LQ fueron similares para las tres matrices. Así, los LD variaron de 1.0 a 462 nM y los LQ de 3.0 a 1540 nM en la orina. Para las muestras de plasma, los LD oscilaron entre 0.9 y 710 nM y los LQ entre 3.0 y 2160 nM. Los LD variaron entre 0.9 y 933 nM y los LQ oscilaron entre 3.0 y 3110 nM en las muestras de heces. En cuanto a la precisión, tanto la precisión intradía como la precisión interdía mostraron una RSD inferior al 15% en las tres matrices analizadas. Las recuperaciones variaron del 70 al 110% en las 3 matrices diferentes. En general, las recuperaciones en las muestras de heces fueron ligeramente más bajas que en el plasma y la orina. En el caso de procianidina B1 y B2, su recuperación fue inferior al 20% en las heces usando los cartuchos OASIS HLB, los cuales tienen un tipo de sorbente de fase inversa que retienen estos compuestos (Xiao et al., 2017). Por otra parte, los metabolitos sulfatados, que son altamente hidrofílicos, mostraron una tasa de recuperación cercana al 70% en las heces, de acuerdo con los valores anteriormente obtenidos por Feliciano et al. (2016) en muestras de plasma y orina. El efecto matriz fue similar en las muestras de orina y plasma y ligeramente más alto en las heces, con un valor medio de -5.68, -4.24 y -8.34, respectivamente. De esta forma, se observó una leve supresión de iones en los análisis de las distintas matrices, de forma similar a otros estudios previos (Gasperotti et al., 2014, Feliciano et al., 2016).

El método fue aplicado para determinar derivados de metabolitos y catabolitos microbianos en muestras de orina, plasma y heces de ratas tras la ingesta de un extracto de vino rico en proantocianidinas. Respecto a las muestras de heces analizadas, los principales metabolitos cuantificados fueron ácido ferúlico (63 nmol), ácido ferúlico-4'-sulfato (78 nmol), ácido 3-(3'-hidroxi-4'-metoxifenil)propiónico (35 nmol), ácido 3'-hidroxifenilacético (228 nmol), ácido 4'-hidroxifenilacético (77 nmol) y ácido 4-hidroxibenzoico (52 nmol). En general, el contenido fecal total de derivados de ácidos fenólicos y ácidos aromáticos fue de 683±152 nmol, equivalente al 4,6% del extracto de proantocianidina ingerido. En el caso de las muestras de plasma, los valores más altos de concentración máxima (Cmax) fueron ácido 3-(3'-hidroxifenil)propiónico (2568 nmol/L), ácido 3-(fenil)propiónico-3'-sulfato (1405 nmol /L), ácido 4-hidroxibenzoico (2660 nmol/L) y ácido hipúrico (433478 nmol/L). Los principales compuestos encontrados en las muestras de orina fueron los derivados de ácidos fenólicos y ácidos aromáticos, con una excreción total de 2567 ± 230 nmol, equivalente al 17.3% de la ingesta. De esta forma, los compuestos mayoritarios excretados fueron ácido ferúlico-4'-sulfato (503 nmol), ácido 3-(3'-metoxi-4'-hidroxifenil)propiónico (97 nmol), ácido 3-(3'-metoxifenil)propiónico-4'-sulfato (106 nmol), ácido 3-(3'-hidroxi-4'-metoxifenil)propiónico (123 nmol), ácido 3-(3'-hidroxifenil)propiónico (150 nmol), ácido 3(fenil)propiónico-3'-sulfato (595 nmol), ácido 4'-hidroxifenilacético (178 nmol) y ácido hipúrico (275 nmol).

En la bibliografía se encuentran varios estudios que han investigado la biodisponibilidad de proantocianidinas en modelos animales (Arola-Arnal et al., 2013; Sano et al., 2003; Tsang et al.,

2005; Prasain et al., 2009). Sin embargo, la mayoría se centraron en el análisis de los monómeros y sus derivados en las muestras biológicas, como plasma u orina, sin tener en cuenta las biotransformaciones de las proantocianidinas mediados por la microbiota colónica. Por el contrario, en un estudio en ratas llevado a cabo por Margalef et al. (2014), se identificaron algunos compuestos derivados de la acción de la microbiota, como el 5-(3',4'-dihidroxifenil)-γ-valerolactona o varios derivados del ácido fenilvalérico.

Además, este método nos permitió identificar por primera vez varios derivados conjugados de fenilvalerolactonas y ácidos fenilvaléricos en las muestras de orina, plasma y heces tras la ingesta del extracto de semilla de uvas rica en proantocianidina (**Tabla 6**).

Tabla 6. Nuevos compuestos identificados en muestras biológicas de ratas tras la ingesta de extracto de vino rico en procianidinas.

Catabolitos identificados (número de isómeros)	Muestras		
5-(Dihidroxifenil)-γ-valerolactona-*O*-glucuronido	O		
5-(Fenil)-γ-valerolactona-*O*-glucuronido-sulfato	O	P	
5-(Metoxifenil)-γ-valerolactona-sulfato (2)	O		H
5-(Metoxifenil)-γ-valerolactona-*O*-glucuronido (2)	O		
5-(Fenil)-γ-valerolactona-4'-*O*-glucuronido	O		
5-(Fenil)-γ-valerolactona-3'-*O*-glucuronido	O	P	
Ácido 5-(3',4'-Dihidroxifenil)-γ-hidroxivalerico	O		H
Ácido 5-(3'-Hidroxifenil)-γ-hidroxivalerico-4'-sulfato	O		H
Ácido 5-(3'-Hidroxifenil)-γ-hidroxivalerico-4'-*O*-glucuronido	O	P	H
Ácido 5-(4'-Hidroxifenil)-γ-hidroxivalerico-3'-*O*-glucuronido	O	P	H
Ácido 5-(3', 4'-Dihidroxifenil)-γ-valerico	O		H
Ácido 5-(Hidroxifenil)-γ-valerico-4'-sulfato	O	P	H

O: Orina. P: Plasma. H: Heces.

Con la identificación de estos compuestos, se propuso una ruta catabólica para las proantocianidinas, la cual se encuentra en el **Capítulo 4** de la presente memoria de tesis.

El **Objetivo 5** se centró en la comparación y evaluación de dos métodos analíticos, un método de cromatografía liquida (HPLC) y un método de cromatografía de gases (GC), ambos acoplados a un detector de masas. Estos métodos se han ambos usado comúnmente en la determinación de ácidos fenólicos en muestras biológicas, principalmente en muestras de orina y heces. Tradicionalmente, la espectrometría de masas (MS) se acopló al GC, no obstante el uso de métodos HPLC acoplados a MS se ha difundido en los últimos años (Kind et al., 2009). Así, la determinación de metabolitos fenólicos en muestras de orina se ha llevado a cabo recientemente tanto por métodos de HPLC-MS (Pereira-Caro et al., 2016; Feliciano et al., 2016;

Sasot et al., 2017; Saenger et al., 2017) como por métodos de GC-MS (Roowi et al., 2010; Wang et al., 2012; Ludwig et al., 2013; Pereira-Caro et al., 2015).

Sin embargo, ambas técnicas presentan diversas ventajas e inconvenientes. Los métodos de GC-MS presentan mejor resolución de picos, no forman aductos de iones moleculares, tienen menor efecto matriz y son métodos más específicos. Además el GC-MS dispone de librerías de metabolitos e índices de retención los cuales pueden ayudar a la identificación de los compuestos a través del espectro de masas (Kind et al., 2009; Scalbert et al., 2009). Del mismo modo, el GC permite identificar y cuantificar pequeñas moléculas polares, las cuales tienen dificultad para ser detectadas por métodos HPLC-MS (Ferreira et al., 2013). Respecto a los métodos de HPLC-MS, entre sus ventajas se encuentran que no es necesario un paso de derivatización de las muestras previo al análisis, el cual suele consumir mucho tiempo y formar derivados poco estables. Además, permite identificar metabolitos conjugados específicos, proporcionando información sobre la transformación y biodisponibilidad de los compuestos fenólicos. En general, el HPLC-MS es una técnica con buenos valores de sensibilidad y reproducibilidad (Scalbert et al., 2009; Pereira-Caro et al., 2016).

Previo al análisis cromatográfico, un factor importante es la preparación de las muestras. Así, las muestras pueden ser extraídas para concentrar los analitos de interés, disminuir las interferencias provocadas por otros compuestos presentes en las muestras y mejorar la sensibilidad, siendo la extracción en fase sólida (SPE) una de las técnicas más empleadas (Savage et al., 2011; van der Hooft et al., 2012; Mosele et al., 2016; Feliciano et al., 2016).

Para ello, se validaron un método de HPLC-HRMS y un método de GC-MS para la determinación de catabolitos derivados de compuestos fenólicos presentes en orina de humanos tras la ingesta de zumo de naranja. Además, se evaluó el proceso de extracción comparando la inyección directa y dos extracciones en SPE usando los cartuchos SDB-L y HBL. Del mismo modo, se determinó la eficiencia del proceso de derivatización con *N*-metil-*N*-(trimetilsilil)trifluoroacetamida (MSTFA). En general, se determinaron un total de 46 compuestos en el método de HPLC y 28 en el método GC en las muestras de orina.

Los métodos fueron validados en términos de especificidad, linealidad, sensibilidad (LD y LQ), precisión (intradía y interdía), recuperación y efecto matriz, de acuerdo con los criterios establecidos por la guía de la FDA (FDA, 2001, 2015). En la **Tabla 7** se muestra la comparación de los métodos cromatográficos validados.

Tabla 7. Comparación de los parámetros de validación de los métodos de cromatografía**.**

Parámetro de Validación	HPLC-HRMS	GC-MS
Nº de analitos validados	46	28
Nº de analitos identificados	35	23
R^2	0.9910 - 0.9999	0.9900 - 0.9999
LD de agliconas (nmoles/L)	20 - 1777	663 – 23964
LQ de agliconas (nmoles/L)	65 - 5924	2209 – 79879
LD de glucurónidos (nmoles/L)	8 - 44	-
LQ de glucurónidos (nmoles/L)	27 - 147	-
LD de sulfatados (nmoles/L)	11 - 46	-
LQ de sulfatados (nmoles/L)	37 - 154	-
Precisión intradía (RSD%)	0.3 - 9.3	1.5 - 9.5
Precisión interdía (RSD%)	1.3 - 12.7	2.2 - 14.2
Efecto matriz (%)	98.7	85.6
Recuperación(Media ± SD)		
Inyección Directa (%)		
Agliconas	97.8 ± 4.3	-
Glucurónidos	102.4 ± 4.1	-
Sulfatados	102.8 ± 3.5	-
SDB-L (%)		
Agliconas	95.0 ± 0.8	78.9 ± 8.4
Glucurónidos	93.8 ± 1.0	-
Sulfatados	11.3 ± 2.9	-
HLB (%)		
Agliconas	94.2 ± 2.6	84.9 ± 5.7
Glucurónidos	95.2 ± 1.2	-
Sulfatados	22.6 ± 10.3	-

R^2: Coeficiente de determinación. RSD: Desviación Estándar Relativa. -: no determinado.

En general, el método de HPLC pudo determinar e identificar un gran número de derivados fenólicos y catabolitos formados por la microbiota colónica, incluidos derivados sulfatados y glucurónidos, a diferencia del método de GC que solo determinó compuestos fenólicos en su forma agliconas. Los resultados de la validación mostraron que los LD y LQ fueron mejores en el método por HPLC en comparación con el GC.

Respecto a los valores de recuperación, tanto los cartuchos SDB-L como los HLB presentaron una buena recuperación para los compuestos fenólicos y sus derivados glucurónidos. En el caso de los derivados sulfatados, los cuales son altamente hidrofílicos, no tuvieron una buena recuperación usando los cartuchos seleccionados. De esta forma, los cartuchos SDB-L, los cuales están compuestos por un copolímero estireno-divinilbenceno, no son útiles para la extracción de estos compuestos. En el caso de los cartuchos HLB, aunque están formados por un sorbente equilibrado hidrofílico-lipofílico, los compuestos muy polares muestran algunos problemas en la extracción, como el ácido clorogénico (Savage et al., 2011) o flavonoles y derivados (Muñoz-González et al., 2014). Por todo ello, la inyección directa es la más

recomendable en relación a la recuperación, ya que evita la pérdida de analitos de interés, principalmente los derivados sulfatados.

En cuanto al proceso de derivatización, se evaluó revirtiendo la reacción de derivatización añadiendo agua. En general, se observó que la mayoría de los analitos estudiados fueron recuperados en torno al 100% tras revertir la derivatización, a excepción de1,3,5-trihidroxifenol. Por otro lado, se comprobó que el proceso de derivatización no provocó una fragmentación del grupo glucurónido o sulfatado de los conjugados, ya que tras revertir la derivatización estos compuestos se recuperaron completamente cuando fueron analizados por HPLC-HRMS.

Del mismo modo que en estudios previos, el ácido 4'-hidroxifenilacético y el ácido hipúrico fueron los analitos que mostraron las concentraciones más altas en todas las muestras analizadas (Pereira-Caro et al., 2016). Además, se identificó el ácido 3-(3'-hidroxi-4'-metoxifenil)hidracrílico, un biomarcador potencial de la ingesta de zumo de naranja procedente de la degradación por las bacterias del colon de la hesperetina (Pereira-Caro et al., 2017).

La aplicación del método en muestras reales mostró que la recuperación de los compuestos fenólicos en forma de agliconas en el método de GC respecto a la inyección directa en el método de LC disminuyó en torno al 20%. Por otro lado, el uso del método de HPLC-MS permitió obtener más información más detallada y completa de los derivados formados del catabolismo de los compuestos fenólicos por las bacterias colónicas. Además, este método fue más sensible y menos laborioso que el método de GC-MS.

CONCLUSIONES

Conclusiones

De los resultados obtenidos durante el desarrollo de la presente Tesis Doctoral se ha llegado a las siguientes conclusiones siguiendo el orden de los objetivos propuestos:

PRIMERA

Para la determinación de las aminas biógenas en bebidas fermentadas (según la revisión bibliográfica de 2010-2016) se emplea principalmente cromatografía líquida con columna C18 en fase reversa y acoplado a detectores de ultravioleta o fluorescencia. Previamente al análisis cromatográfico, se suelen emplear diferentes tipos de reactivos derivatizantes para mejorar la sensibilidad y selectividad analítica, obteniéndose unos valores de LD y LQ adecuados para su aplicación en seguridad alimentaria, proceso de producción o investigación de microbiología alimentaria.

SEGUNDA

Se ha validado con éxito un método de HPLC acoplado a un detector de fotodiodo y usando DEEMM como reactivo derivatizante para la determinación de aminas biógenas, aminoácidos y amonio en puré de fresa y su fermentado glucónico, obteniendo valores adecuados de linealidad, sensibilidad y precisión. Se determinaron un total de 31 compuestos, incluidos 22 aminoácidos, 8 aminas biógenas e ion amonio. Se ha demostrado su utilidad para el análisis de rutina de estos compuestos nitrogenados.

TERCERA

Los fermentados glucónicos obtenidos mediante cultivo superficial no contienen aminas biógenas por encima de los LD del método. El perfil de aminoácidos permitió la discriminación de las bebidas según la cepa de bacteria acética responsable de la fermentación. *G. japonicus* fue la cepa seleccionada, ya que mostró mayor actividad durante la fermentación consumiendo la glucosa más eficiente.

CUARTA

Los fermentados glucónicos obtenidos mediante cultivo sumergido no contienen aminas biógenas por encima de los LD del método. Estos fermentados son, por tanto, seguros para la elaboración de productos de consumo humano. Además, esta fermentación no provocó cambios significativos en el perfil de aminoácidos.

QUINTA

Se ha validado con éxito un método de HPLC acoplado a un detector de fluorescencia y usando AQC como reactivo derivatizante para la determinación de aminas biógenas en vinagre, obteniendo valores adecuados de selectividad, linealidad, sensibilidad, precisión y recuperación. Se determinaron un total de 9 aminas biógenas. Este método ha mostrado ser útil su utilidad para el análisis de rutina de aminas biógenas en muestras de vinagre.

SEXTA

La concentración de aminas biógenas en los vinagres fue menor que la encontrada en los vinos. La putrescina y la histamina fueron las aminas biógenas que presentaron mayor concentración. Entre los vinagres analizados, los balsámicos y el Pedro Ximénez presentaron los niveles más altos de aminas biógenas, mientras que el vinagre de manzana presentaron las concentraciones más bajas. Estas diferencias en el perfil de aminas biógenas de los vinagres analizados se confirmaron/reflejaron en el Análisis de Componentes Principales (PCA), ya que considerando como variables las aminas biógenas, fue posible separar los diferentes tipos de vinagres, exceptuando los vinagres de vino tinto.

SÉPTIMA

La concentración de aminas biógenas no cambió significativamente en botellas de vino abiertas y conservadas en diferentes condiciones de almacenamiento (temperatura y tapón). Aunque el perfil de aminas biógenas cambió ligeramente a lo largo del tiempo en los tres tipos de vinos, la concentración final de estas biomoléculas en los vinos estaba principalmente determinada por sus cantidades iniciales. El Análisis Discriminante Lineal (LDA) confirmó que las muestras se agruparon según su tiempo de almacenamiento, independientemente de las condiciones de almacenamiento.

OCTAVA

Se ha desarrollado y validado con éxito una metodología analítica mediante el empleo de cromatografía líquida acoplada a un detector de espectrometría de masas de alta resolución para la caracterización y cuantificación de compuestos fenólicos y sus metabolitos en muestras de orina, plasma y heces de ratas obteniendo valores adecuados de especificidad, linealidad, sensibilidad, precisión, recuperación y efecto matriz.

NOVENA

La metodología propuesta se ha aplicado para la caracterización y determinación de un total de 34 metabolitos, incluidos 3 flavanoles, 4 derivados de valerolactonas y 27 ácidos fenólicos y

ácidos aromáticos en muestras de orina, plasma y heces de ratas tras la ingesta por éstas de un extracto de semilla de uva rica en proantocianidinas. Los resultados han mostrado que estos compuestos fenólicos son altamente metabolizados por las bacterias colónicas. Adicionalmente la aplicación de este método cromatográfico ha permitido la identificación por primera vez de 8 derivados conjugados de fenilvalerolactonas y 6 ácidos fenilvaléricos, siendo los conjugados sulfatados los principales metabolitos identificados tras la ingesta de este extracto en ratas. Los derivados fenólicos mayoritarios en orina fueron el ácido 3(fenil)propiónico-3'-sulfato y el ácido ferúlico-4'-sulfato y en heces fue el ácido 3'-hidroxifenilacético. En general, los derivados de ácidos fenólicos y ácidos aromáticos encontrados en las muestras de orina y heces equivalen al 17.3% y 4.6% del extracto de procianidina ingerido, respectivamente. La concentración máxima de los metabolitos fenólicos en plasma fueron el ácido hipúrico, el ácido 4-hidroxibenzoico y el ácido 3-(3'-hidroxifenil)propiónico.

DÉCIMA

Se han validado con éxito un método de HPLC acoplado a un detector de espectrometría de masas de alta resolución y un método de GC acoplado a un detector de espectrometría de masas para la determinación compuestos fenólicos y sus metabolitos en muestras de orina tras el consumo de zumo de naranja por sujetos sanos, obteniendo valores adecuados de especificidad, linealidad, sensibilidad, precisión, recuperación y efecto matriz. Además, se han evaluado diferentes sistemas de purificación de muestras previo al análisis por cromatografía, siendo la inyección directa de muestra de orina la que presentó mejores resultados de recuperación frente a la obtenida empleando el sistema SPE con cartuchos (SBD-L y HLB), principalmente para los compuestos muy hidrofílicos como los derivados sulfatados.

UNDÉCIMA

Tanto el método por cromatografía líquida (HPLC) como por cromatografía gaseosa (GC) son adecuados para ser aplicados en estudios de biodisponibilidad centrados en el estudio del perfil de compuestos fenólicos generados por degradación microbiana de los polifenoles tras su ingesta. No obstante, existen diversas limitaciones a tener en cuenta sobre todo en el método GC. A nivel cualitativo, las principales diferencias entre ambos métodos fueron en el número de metabolitos identificados. Por el método HPLC se determinaron un total de 46 compuestos fenólicos, incluyendo derivados glucurónidos y sulfatados, mientras que por el método GC sólo se pudieron identificar 28 ácidos fenólicos encontrándose todos ellos en su forma libre. A nivel cuantitativo existen diferencias entre ambas técnicas analíticas, siendo el método HPLC el obtuvo mejores resultados y una información más detallada y completa de los metabolitos presentes.

BIBLIOGRAFÍA

Bibliografía

Abad-García, B., Garmón-Lobato, S., Berrueta, L. A., Gallo, B., & Vicente, F. (2012). On line characterization of 58 phenolic compounds in Citrus fruit juices from Spanish cultivars by high-performance liquid chromatography with photodiode-array detection coupled to electrospray ionization triple quadrupole mass spectrometry. *Talanta*, *99*, 213–224.

Acosta-Estrada, B. A., Gutiérrez-Uribe, J. A., & Serna-Saldívar, S. O. (2014). Bound phenolics in foods, a review. *Food Chemistry*, *152*, 46–55.

Actis-Goreta, L., Lévèques, A., Giuffrida, F., Romanov-Michailidis, F., Viton, F., Barron, D., … Dionisi, F. (2012). Elucidation of (-)-epicatechin metabolites after ingestion of chocolate by healthy humans. *Free Radical Biology and Medicine*, *53*, 787–795.

Actis-Goretta, L., Lévèques, A., Rein, M., Teml, A., Schäfer, C., Hofmann, U., … Williamson, G. (2013). Intestinal absorption, metabolism, and excretion of (-)-epicatechin in healthy humans assessed by using an intestinal perfusion technique. *American Journal of Clinical Nutrition*, *98*, 924–933.

Allison, C., & Macfarlane, G. T. (1989). Influence of pH, nutrient availability, and growth rate on amine production by Bacteroides fragilis and Clostridium perfringens. *Applied and Environmental Microbiology*, *55*, 2894–2898.

Almeida, C., Fernandes, J. O., & Cunha, S. C. (2012). A novel dispersive liquid–liquid microextraction (DLLME) gas chromatography-mass spectrometry (GC–MS) method for the determination of eighteen biogenic amines in beer. *Food Control*, *25*(1), 380–388.

Álvarez, M. A., & Moreno-Arribas, M. V. (2014). The problem of biogenic amines in fermented foods and the use of potential biogenic amine-degrading microorganisms as a solution. *Trends in Food Science & Technology*, *39*(2), 146–155.

Álvarez-Fernández, M. A., Hornedo-Ortega, R., Cerezo, A. B., Troncoso, A. M., & García-Parrilla, M. C. (2014). Non-anthocyanin phenolic compounds and antioxidant activity of beverages obtained by gluconic fermentation of strawberry. *Innovative Food Science & Emerging Technologies*, *26*, 469-481.

Ancín-Azpilicueta, C., González-Marco, A., & Jiménez-Moreno, N. (2008). Current knowledge about the presence of amines in wine. *Critical Reviews in Food Science and Nutrition*, *48*, 257–275.

Andreasen, M. F., Kroon, P. A., Williamson, G., & Garcia-Conesa, M.-T. (2001). Intestinal

release and uptake of phenolic antioxidant diferulic acids. *Free Radical Biology and Medicine*, *31*(3), 304–314.

AOAC. (2007). Association of Analytical Communities (AOAC). How to meet ISO 17025 requirements for methods verification, 2007.

Arola-Arnal, A., Oms-Oliu, G., Crescenti, A., Bas, J.M., Ras, M.R., Arola, L., Caimari, A. Distribution of grape seed flavanols and their metabolites in pregnant rats and their foetuses. *Molecular Nutrition and Food Research*, 2013, 57, 1741-1752.

Askar, A., & Treptow, H. (1986). Biogene Amine in Lebensmitteln — Vorkommen, Bedeutung und Bestimmung. *Verlag Eugen Ulmer KG*.

Bardócz, S. (1995). Polyamines in food and their consequences for food quality and human health. *Trends in Food Science & Technology*, *6*(10), 341–346.

Barron, D., Smarrito-Menozzi, C., Fumeaux, R., & Viton, F. (2012). Synthesis of dietary phenolic metabolites and isotopicallylabeled dietary phenolics. In Spencer JPE and Crozier A (Ed.), *Bioavailability and Function of Flavonoids* (pp. 233–280). CRC Press, Boca Raton.

Basheer, C., Wong, W., Makahleh, A., Tameem, A. A., Salhin, A., Saad, B., & Lee, H. K. (2011). Hydrazone-based ligands for micro-solid phase extraction-high performance liquid chromatographic determination of biogenic amines in orange juice. *Journal of Chromatography. A*, *1218*(28), 4332–9.

Bauza, T., Blaise, A., Daumas, F., & Cabanis, J. C. (1995). Determination of biogenic amines and their precursor amino acids in wines of the Vallé du Rhone by high-performance liquid chromatography with precolumn derivatization and fluorimetric detection. *Journal of Chromatography A*, *707*(2), 373–379.

Bauza, T., Kelly, M. T., & Blaise, A. (2007). Study of polyamines and their precursor amino acids in Grenache noir and Syrah grapes and wine of the Rhone Valley. *Food Chemistry*, *105*(1), 405–413.

Bedia Erim, F. (2013). Recent analytical approaches to the analysis of biogenic amines in food samples. *TrAC Trends in Analytical Chemistry*, *52*, 239–247.

Berbegal, C., Benavent-Gil, Y., Navascués, E., Calvo, A., Albors, C., Pardo, I., & Ferrer, S. (2017). Lowering histamine formation in a red Ribera del Duero wine (Spain) by using an

indigenous O. oeni strain as a malolactic starter. *International Journal of Food Microbiology*, *244*, 11–18.

Blackwell, B., Marley, E., Price, J., & Taylor, D. (1967). Hypertensive interactions between monoamine oxidase inhibitors and foodstuffs. *British Journal of Psychiatry*, *113*, 349–365.

Borges, G., Lean, M. E. J., Roberts, S. A., & Crozier, A. (2013). Bioavailability of dietary (poly)phenols: a study with ileostomists to discriminate between absorption in small and large intestine. *Food & Function*, *4*, 754–762.

Borges, G., Mullen, W., Mullan, A., Lean, M. E. J., Roberts, S. A., & Crozier, A. (2010). Bioavailability of multiple components following acute ingestion of a polyphenol-rich juice drink. *Molecular Nutrition and Food Research*, *54*, S268-277.

Borges, G., Ottaviani, J. I., van der Hooft, J. J. J., Schroeter, H., & Crozier, A. (2017). Absorption, metabolism, distribution and excretion of (-)-epicatechin: A review of recent findings. *Molecular Aspects of Medicine*, *In Press*.

Boto-Ordóñez, M., Urpi-Sarda, M., Queipo-Ortuño, M. I., Corella, D., Tinahones, F. J., Estruch, R., & Andres-Lacueva, C. (2013). Microbial metabolomic fingerprinting in urine after regular dealcoholized red wine consumption in humans. *Journal of Agricultural and Food Chemistry*, *61*, 9166–9175.

Bravo, L. (1998). Lead Review Article Polyphenols: Chemistry, Dietary Sources, Metabolism, and Nutritional Significance. *Nutritions Reviews*, *56*, 317–333.

Bredsdorff, L., Nielsen, I. L. F., Rasmussen, S. E., Cornett, C., Barron, D., Bouisset, F., … Williamson, G. (2010). Absorption, conjugation and excretion of the flavanones, naringenin and hesperetin from α-rhamnosidase-treated orange juice in human subjects. *British Journal of Nutrition*, *103*(11), 1602–1609.

Brindani, N., Mena, P., Calani, L., Benzie, I., Choi, S.-W., Brighenti, F., … Del Rio, D. (2017). Synthetic and analytical strategies for the quantification of phenyl-γ-valerolactone conjugated metabolites in human urine. *Molecular Nutrition & Food Research*, *61*(9), 1700077.

Busto, O., Guasch, J., & Borrull, F. (1995). Improvement of a solid-phase extraction method for determining biogenic amines in wines. *Journal of Chromatography A*, *718*(2), 309–317.

Calani, L., Del Rio, D., Luisa Callegari, M., Morelli, L., & Brighenti, F. (2012). Updated

bioavailability and 48 h excretion profile of flavan-3-ols from green tea in humans. *International Journal of Food Sciences and Nutrition*, *63*(5), 513–521.

Callejón, R. M., Tesfaye, W., Torija, M. J., Mas, A., Troncoso, A. M., & Morales, M. L. (2008). HPLC determination of amino acids with AQC derivatization in vinegars along submerged and surface acetifications and its relation to the microbiota. *European Food Research and Technology*, *227*(1), 93–102.

Callejón, R. M., Troncoso, A. M., & Morales, M. L. (2010). Determination of amino acids in grape-derived products: a review. *Talanta*, *81*(4), 1143–1152.

Cardona-Gálvez, M., & González-Domínguez, A. (2005). Intoxicación histamínica o escombroidosis en pescados. *Alimentaria*, *265*, 68–73.

Chan, E. C. Y., Pasikanti, K. K., & Nicholson, J. K. (2011). Global urinary metabolic profiling procedures using gas chromatography–mass spectrometry. *Nature Protocols*, *6*(10), 1483–1499.

Chattopadhyay, M. K., White Tabor, C., & Tabor, H. (2003). Polyamines protect Escherichia coli cells from the toxic effect of oxygen. *Proceedings of the National Academy of Sciences of the United States of America*, *100*, 2261–2265.

Chinnici, F., Durán-Guerrero, E., & Riponi, C. (2016). Discrimination of some European vinegars with protected denomination of origin as a function of their amino acid and biogenic amine content. *Journal of the Science of Food and Agriculture*, *96*(11), 3762–3771.

Chow, H.-H. S., Hakim, I. A., Vining, D. R., Crowell, J. A., Ranger-Moore, J., Chew, W. M., … Alberts, D. S. (2005). Effects of Dosing Condition on the Oral Bioavailability of Green Tea Catechins after Single-Dose Administration of Polyphenon E in Healthy Individuals. *Clinical Cancer Research*, *11*(12), 4627–4633.

Comisión Europea. (2002). Reglamento (CE) nº 178/2002 Del Parlamento Europeo y Del Consejo.

Costantini, A., Pietroniro, R., Doria, F., Pessione, E., & Garcia-Moruno, E. (2013). Putrescine production from different amino acid precursors by lactic acid bacteria from wine and cider. *International Journal of Food Microbiology*, *165*(1), 11–17.

Crozier, A. (2013). Absorption, metabolism, and excretion of (-)-epicatechin in humans: an evaluation of recent findings. *The American Journal of Clinical Nutrition*, *98*(4), 861–2.

Crozier, A., Del Rio, D., & Clifford, M. N. (2010). Bioavailability of dietary flavonoids and phenolic compounds. *Molecular Aspects of Medicine*, *31*(6), 446–467.

Czank, C., Cassidy, A., Zhang, Q., Morrison, D. J., Preston, T., Kroon, P. A., … Kay, C. D. (2013). Human metabolism and elimination of the anthocyanin, cyanidin-3-glucoside: a 13C-tracer study. *American Journal of Clinical Nutrition*, *97*(5), 995–1003.

Day, A. J., Cañada, F. J., Díaz, J. C., Kroon, P. A., Mclauchlan, R., Faulds, C. B., … Williamson, G. (2000). Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. *FEBS Letters*, *468*(2–3), 166–170.

De Ferrars, R. M., Czank, C., Saha, S., Needs, P. W., Zhang, Q., Raheem, K. S., … Kay, C. D. (2014). Methods for isolating, identifying, and quantifying anthocyanin metabolites in clinical samples. *Analytical Chemistry*, *86*, 10052–10058.

De Oliveira, D. M., Pinto, C. B., Sampaio, G. R., Yonekura, L., Catharino, R. R., & Bastos, D. H. M. (2013). Development and validation of methods for the extraction of phenolic acids from plasma, urine, and liver and analysis by UPLC-MS. *Journal of Agricultural and Food Chemistry*, *61*, 6113–6121.

Del Prete, V., Costantini, A., Cecchini, F., Morassut, M., & Garcia-Moruno, E. (2009). Occurrence of biogenic amines in wine: The role of grapes. *Food Chemistry*, *112*, 474–481.

Del Rio, D., Costa, L. G., Lean, M. E. J., & Crozier, A. (2010). Polyphenols and health: What compounds are involved? *Nutrition, Metabolism and Cardiovascular Diseases*, *20*, 1–6.

Del Rio, D., Rodriguez-Mateos, A., Spencer, J. P. E., Tognolini, M., Borges, G., & Crozier, A. (2013). Dietary (Poly)phenolics in Human Health: Structures, Bioavailability, and Evidence of Protective Effects Against Chronic Diseases. *Antioxidants & Redox Signaling*, *18*, 1818–1892.

Di Martino, M. L., Campilongo, R., Casalino, M., Micheli, G., Colonna, B., & Prosseda, G. (2013). Polyamines: Emerging players in bacteria-host interactions. *International Journal of Medical Microbiology*, *303*, 484–491.

Donovan, J., Manach, C., Faulks, R., & Kroon, P. (2006). Absorption and metabolism of dietary plant secondary metabolites. In Crozier & A. H. A, Clifford MN (Eds.), *plant secondary metabolites. Occurrence, structure and role in the human diet.* (pp. 303–351). Blackwell Publishing, Oxford.

Ducros, V., Ruffieux, D., Belva-Besnet, H., Fraipont, F. de, Berger, F., & Favier, A. (2009). Determination of dansylated polyamines in red blood cells by liquid chromatography-tandem mass spectrometry. *Analytical Biochemistry*, *390*, 46–51.

Duda-Chodak, A., Tarko, T., Satora, P., & Sroka, P. (2015). Interaction of dietary compounds, especially polyphenols, with the intestinal microbiota: a review. *European Journal of Nutrition*, *54*, 325–341.

Dufour, C., Loonis, M., Delosière, M., Buffière, C., Hafnaoui, N., Santé-Lhoutellier, V., & Rémond, D. (2018). The matrix of fruit and vegetables modulates the gastrointestinal bioaccessibility of polyphenols and their impact on dietary protein digestibility. *Food Chemistry*, *240*, 314–322.

Erk, T., Renouf, M., Williamson, G., Melcher, R., Steiling, H., & Richling, E. (2014). Absorption and isomerization of caffeoylquinic acids from different foods using ileostomist volunteers. *European Journal of Nutrition*, *53*(1), 159–166.

Eurachem. (1998). Eurachem. The fitness for purpose of analytical methods. A laboratory guide to method validation an related topics. Eurachem Guide, 1998.

Eurachem. (2014). Eurachem Guide: The fitness for purpose of analytical methods. A laboratory guide to method validation an related topics (2nd Ed.). Eurachem Guide, 2014.

Fang, Z., Zhao, Y., Warner, R. D., & Johnson, S. K. (2017). Active and intelligent packaging in meat industry. *Trends in Food Science and Technology*, *61*, 60–71.

Faria, A., Pestana, D., Azevedo, J., Martel, F., de Freitas, V., Azevedo, I., … Calhau, C. (2009). Absorption of anthocyanins through intestinal epithelial cells - Putative involvement of GLUT2. *Molecular Nutrition & Food Research*, *53*(11), 1430–1437.

FDA. (2001). Food and Drug Administration (FDA). Guidance for Industry: Bioanalytical Method Validation; US Department of Health and Human Service, Food and Drug Administration, Center for Drug Evaluation and Research: Rockville, MD, 2001.

FDA. (2015). Food and Drug Administration (FDA). Acceptance Criteria for Confirmation of Identity of Chemical Residues using Exact Mass Data for the FDA Foods and Veterinary Medicine Program, 2015.

Feliciano, R. P., Mecha, E., Bronze, M. R., & Rodriguez-Mateos, A. (2016). Development and validation of a high-throughput micro solid-phase extraction method coupled with ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry for

rapid identification and quantification of phenolic metabolites in human plasma and urine. *Journal of Chromatography A*, *1464*, 21–31.

Feng, C., Teuber, S., & Gershwin, M. E. (2016). Histamine (Scombroid) Fish Poisoning: a Comprehensive Review. *Clinical Reviews in Allergy and Immunology*, *50*, 64–69.

Fernández-García, E., Carvajal-Lérida, I., & Pérez-Gálvez, A. (2009). In vitro bioaccessibility assessment as a prediction tool of nutritional efficiency. *Nutrition Research*, *29*(11), 751–760.

Fernández, M., Linares, D. M., & Alvarez, M. A. (2004). Sequencing of the tyrosine decarboxylase cluster of Lactococcus lactis IPLA 655 and the development of a PCR method for detecting tyrosine decarboxylating lactic acid bacteria. *Journal of Food Protection*, *67*(11), 2521–2529.

Fernandez de Palencia, P., Fernandez, M., Mohedano, M. L., Ladero, V., Quevedo, C., Alvarez, M. A., & Lopez, P. (2011). Role of Tyramine Synthesis by Food-Borne Enterococcus durans in Adaptation to the Gastrointestinal Tract Environment. *Applied and Environmental Microbiology*, *77*(2), 699–702.

Ferreira, A. M. C., Laespada, M. E. F., Pavón, J. L. P., & Cordero, B. M. (2013). In situ aqueous derivatization as sample preparation technique for gas chromatographic determinations. *Journal of Chromatography A*, *1296*, 70-83.

García-Marino, M., Trigueros, Á., & Escribano-Bailón, T. (2010). Influence of oenological practices on the formation of biogenic amines in quality red wines. *Journal of Food Composition and Analysis*, *23*(5), 455–462.

García-Villar, N., Hernández-Cassou, S., & Saurina, J. (2009). Determination of biogenic amines in wines by pre-column derivatization and high-performance liquid chromatography coupled to mass spectrometry. *Journal of Chromatography A*, *1216*(36), 6387–6393.

Gasperotti, M., Masuero, D., Guella, G., Mattivi, F., & Vrhovsek, U. (2014). Development of a targeted method for twenty-three metabolites related to polyphenol gut microbial metabolism in biological samples, using SPE and UHPLC-ESI-MS/MS. *Talanta*, *128*, 221–230.

Geddes, R. D., Wang, X., Yomano, L. P., Miller, E. N., Zheng, H., Shanmugam, K. T., & Ingram, L. O. (2014). Polyamine transporters and polyamines increase furfural tolerance during xylose fermentation with ethanologenic Escherichia coli strain LY180. *Applied and*

Environmental Microbiology, *80*, 5955–5964.

Gee, J. M., DuPont, M. S., Day, A. J., Plumb, G. W., Williamson, G., & Johnson, I. T. (2000). Intestinal transport of quercetin glycosides in rats involves both deglycosylation and interaction with the hexose transport pathway. *The Journal of Nutrition*, *130*(11), 2765–71.

Gil, Á. (2017). *Tratado de Nutrición. Composición y Calidad de los alimentos*.

Gillman, P. K. (2011). Advances Pertaining to the Pharmacology and Interactions of Irreversible Nonselective Monoamine Oxidase Inhibitors. *Journal of Clinical Psychopharmacology*, *31*, 66–74.

Gómez-Alonso, S., Hermosín-Gutiérrez, I., & García-Romero, E. (2007). Simultaneous HPLC analysis of biogenic amines, amino acids, and ammonium ion as aminoenone derivatives in wine and beer samples. *Journal of Agricultural and Food Chemistry*, *55*(3), 608–613.

Gómez-Juaristi, M., Martínez-López, S., Sarria, B., Bravo, L., & Mateos, R. (2018). Absorption and metabolism of yerba mate phenolic compounds in humans. *Food Chemistry*, *240*, 1028–1038.

González-Marco, A., & Ancín-Azpilicueta, C. (2006). Influence of lees contact on evolution of amines in Chardonnay wine. *Journal of Food Science*, *71*, 544–548.

Goodrich, K. M., & Neilson, A. P. (2014). Simultaneous UPLC-MS/MS analysis of native catechins and procyanidins and their microbial metabolites in intestinal contents and tissues of male Wistar Furth inbred rats. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *958*, 63–74.

Green, J. M. (1996). A Practical Guide to Analytical Method Validation. *Analitycal Chemistry New & Features*, *May 1*, 305–309.

Griswold, A. R., Jameson-Lee, M., & Burne, R. A. (2006). Regulation and physiologic significance of the agmatine deiminase system of Streptococcus mutans UA159. *Journal of Bacteriology*, *188*, 834–841.

Guo, X., Guan, X., Wang, Y., Li, L., Wu, D., Chen, Y., … Xiao, D. (2015). Reduction of biogenic amines production by eliminating the PEP4 gene in Saccharomyces cerevisiae during fermentation of Chinese rice wine. *Food Chemistry*, *178*, 208–211.

Gupta, K., Dey, A., & Gupta, B. (2013). Plant polyamines in abiotic stress responses. *Acta Physiologiae Plantarum*, *35*, 2015–2036.

Gustavo González, A., & Ángeles Herrador, M. (2007). A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles. *TrAC - Trends in Analytical Chemistry*, *26*, 227–238.

Handa, A. K., & Mattoo, A. K. (2010). Differential and functional interactions emphasize the multiple roles of polyamines in plants. *Plant Physiology and Biochemistry*, *48*, 540–546.

Heising, J. K., van Boekel, M. A. J. S., & Dekker, M. (2015). Simulations on the prediction of cod (*Gadus morhua*) freshness from an intelligent packaging sensor concept. *Food Packaging and Shelf Life*, *3*, 47–55.

Henning, S. M., Niu, Y., Liu, Y., Lee, N. H., Hara, Y., Thames, G. D., … Heber, D. (2005). Bioavailability and antioxidant effect of epigallocatechin gallate administered in purified form versus as green tea extract in healthy individuals. *The Journal of Nutritional Biochemistry*, *16*(10), 610–616.

Hernández-Cassou, S., & Saurina, J. (2011). Derivatization strategies for the determination of biogenic amines in wines by chromatographic and electrophoretic techniques. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, *879*(17–18), 1270–1281.

Hernández-Orte, P., Ibarz, M. J., Cacho, J., & Ferreira, V. (2003). Amino acid determination in grape juices and wines by HPLC using a modification of the 6-Aminoquinolyl-N-Hydroxysuccinimidyl Carbamate (AQC) method. *Chromatographia*, *58*, 29–35.

Hernández-Orte, P., Lapeña, A. C., Peña-Gallego, A., Astrain, J., Baron, C., Pardo, I., … Ferreira, V. (2008). Biogenic amine determination in wine fermented in oak barrels: Factors affecting formation. *Food Research International*, *41*, 697–706.

Hernández-Orte, P., Peña-Gallego, A., Ibarz, M. J., Cacho, J., & Ferreira, V. (2006). Determination of the biogenic amines in musts and wines before and after malolactic fermentation using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate as the derivatizing agent. *Journal of Chromatography A*, *1129*(2), 160-164.

Hornedo-Ortega, R., Álvarez-Fernández, M. A., Cerezo, A. B., Troncoso, A. M., & García-Parrilla, M. C. (2016). Influence of storage conditions on the anthocyanin profile and colour of an innovative beverage elaborated by gluconic fermentation of strawberry. *Journal of Functional Foods*, *23*, 198-209.

Hu, Z., Li, L., Yuan, Y., & Yue, T. (2014). Ultrasensitive and simultaneous determination of twenty-one amino acids and amines in culture media, red wine and beer. *Food Chemistry*,

158, 56–65.

Huber, L. (2010). *Validation of analytical methods.* Agilent Technologies.

ICH. (1995). International Conference on Harmonization (ICH). Validation of analytical procedures: Definition and terminology, Geneva 1995, in 2005 incorporated in Q2(R1).

ICH. (1996). International Conference on Harmonization (ICH). Validation of Analytical Procedures: Metodology, Geneva 1996, in 2005 incorporated in Q2(R1).

Ignat, I., Volf, I., & Popa, V. I. (2011). A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chemistry*, *126*(4), 1821–1835.

International Organisation of Vine and Wine (OIV). (n.d.). Methods of analysis. Retrieved March 31, 2016, from http://www.oiv.int/en/

ISO. (2005). International Organization for Standardization (ISO). ISO 17025, general requirements for the competence of testing and calibration laboratories 2005.

Iswaldi, I., Arráez-Román, D., Gómez-Caravaca, A. M., Contreras, M. del M., Uberos, J., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2013). Identification of polyphenols and their metabolites in human urine after cranberry-syrup consumption. *Food and Chemical Toxicology*, *55*, 484–492.

IUPAC. (1997). Internacional Union of Pure and Apply Chemistry (IUPAC). Compendium of Analytical Nomenclature, Definitive Rules 1997, 3.ª Ed., Blackwell Science, Oxford, 1998.

IUPAC. (2002). Internacional Union of Pure and Apply Chemistry (IUPAC). IUPAC. Technical report, harmonized guidelines for single-laboratory validation of methods of analysis. Pure Applications of Chemistry 2002; 74 (5): 835-855.

Jaganath, I. B., Mullen, W., Edwards, C. A., & Crozier, A. (2006). The relative contribution of the small and large intestine to the absorption and metabolism of rutin in man. *Free Radical Research*, *40*, 1035–1046.

Jarisch, R. (2004). *Histamin-Intoleranz, Histamin und Seekrankheit*. Thieme.

Jia, S., Kang, Y. P., Park, J. H., Lee, J., & Kwon, S. W. (2011). Simultaneous determination of 23 amino acids and 7 biogenic amines in fermented food samples by liquid chromatography/quadrupole time-of-flight mass spectrometry. *Journal of Chromatography A*, *1218*(51), 9174–9182.

Jiménez-Bremont, J. F., Marina, M., Guerrero-González, M. de la L., Rossi, F. R., Sánchez-Rangel, D., Rodrí-guez-Kessler, M., … Gárriz, A. (2014). Physiological and molecular implications of plant polyamine metabolism during biotic interactions. *Frontiers in Plant Science*, *5*(95), 1–14.

Kalač, P. (2014). Health effects and occurrence of dietary polyamines: A review for the period 2005-mid 2013. *Food Chemistry*, *161*, 27–39.

Kalač, P., & Krausová, P. (2005). A review of dietary polyamines: Formation, implications for growth and health and occurrence in foods. *Food Chemistry*, *90*, 219–230.

Karovicova, J., & Kohajdova, Z. (2005). Biogenic Amines in Food. *Chemical Papers*, *36*(34), 479–484.

Kay, C. D., Pereira-Caro, G., Ludwig, I. A., Clifford, M. N., & Crozier, A. (2017). Anthocyanins and Flavanones Are More Bioavailable than Previously Perceived: A Review of Recent Evidence. *Annual Review of Food Science and Technology*, *8*, 1–26.

Kelly, M. T., Blaise, A., & Larroque, M. (2010). Rapid automated high performance liquid chromatography method for simultaneous determination of amino acids and biogenic amines in wine, fruit and honey. *Journal of Chromatography. A*, *1217*(47), 7385–92.

Kind, T., Wohlgemuth, G., Lee, D. Y., Lu, Y., Palazoglu, M., Shahbaz, S., & Fiehn, O. (2009). FiehnLib: mass spectral and retention index libraries for metabolomics based on quadrupole and time-of-flight gas chromatography/mass spectrometry. *Analytical chemistry*, *81*(24), 10038-10048.

Kirschbaum, J., Meier, A., & Brückner, H. (1999). Determination of biogenic amines in fermented beverages and vinegars by pre-column derivatization withpara-nitrobenzyloxycarbonyl chloride (PNZ-Cl) and reversed-phase LC. *Chromatographia*, *49*(3–4), 117–124.

Konakovsky, V., Focke, M., Hoffmann-Sommergruber, K., Schmid, R., Scheiner, O., Moser, P., … Hemmer, W. (2011). Levels of histamine and other biogenic amines in high-quality red wines. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment*, *28*, 408–416.

Konishi, Y., Zhao, Z., & Shimizu, M. (2006). Phenolic acids are absorbed from the rat stomach with different absorption rates. *Journal of Agricultural and Food Chemistry*, *54*, 7539–7543.

Kroon, P. A., Clifford, M. N., Crozier, A., Day, A. J., Donovan, J. L., Manach, C., & Williamson, G. (2004). How should we assess the effects of exposure to dietary polyphenols in vitro? *The American Journal of Clinical Nutrition*, *80*(1), 15–21.

Kummasook, A., Cooper, C. R., Sakamoto, A., Terui, Y., Kashiwagi, K., & Vanittanakom, N. (2013). Spermidine is required for morphogenesis in the human pathogenic fungus, Penicillium marneffei. *Fungal Genetics and Biology*, *58–59*, 25–32.

Ladero, V., Calles-Enriquez, M., Fernandez, M., & A. Alvarez, M. (2010). Toxicological Effects of Dietary Biogenic Amines. *Current Nutrition & Food Science*, *6*(2), 145–156.

Lafay, S., & Gil-Izquierdo, A. (2008). Bioavailability of phenolic acids. *Phytochemistry Reviews*, *7*, 301–311.

Landete, J. M., Ferrer, S., Polo, L., & Pardo, I. (2005). Biogenic amines in wines from three Spanish regions. *Journal of Agricultural and Food Chemistry*, *53*, 1119–1124.

Lee, M.-J., Maliakal, P., Chen, L., Meng, X., Bondoc, F. Y., Prabhu, S., … Yang, C. S. (2002). Pharmacokinetics of tea catechins after ingestion of green tea and (-)-epigallocatechin-3-gallate by humans: formation of different metabolites and individual variability. *Cancer Epidemiology, Biomarkers & Prevention : A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology*, *11*, 1025–1032.

Lee, Y. H., Kim, B. H., Kim, J. H., Yoon, W. S., Bang, S. H., & Park, Y. K. (2007). CadC has a global translational effect during acid adaptation in Salmonella enterica serovar Typhimurium. *Journal of Bacteriology*, *189*(6), 2417–2425.

Lehane, L., & Olley, J. (2000). Histamine fish poisoning revisited. *International Journal of Food Microbiology*, *58*, 1–37.

LGC. (2003). Laboratory of the Government Chemistry (LGC). In-house method validation: A guide for chemical laboratories, 2003.

Li, J. S., Wang, H., Huang, K. J., & Zhang, H. S. (2006). Determination of biogenic amines in apples and wine with 8-phenyl-(4-oxy-acetic acid N-hydroxysuccinimide ester)-4, 4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene by high performance liquid chromatography. *Analytica Chimica Acta*, *575*, 255–261.

Linares, D. M., Del Río, B., Ladero, V., Martínez, N., Fernández, M., Martín, M. C., & Álvarez, M. A. (2012). Factors influencing biogenic amines accumulation in dairy products.

Frontiers in Microbiology, *3*, 1–10.

Linares, D. M., del Rio, B., Redruello, B., Ladero, V., Martin, M. C., Fernandez, M., … Alvarez, M. A. (2016). Comparative analysis of the in vitro cytotoxicity of the dietary biogenic amines tyramine and histamine. *Food Chemistry*, *197*, 658–663.

Linares, D. M., Martín, M., Ladero, V., Alvarez, M. A., & Fernández, M. (2011). Biogenic Amines in Dairy Products. *Critical Reviews in Food Science and Nutrition*, *51*(7), 691–703.

Lonvaud-Funel, A. (2001). Biogenic amines in wines: role of lactic acid bacteria. *FEMS Microbiology Letters*, *199*(1), 9–13.

Lozanov, V., Petrov, S., & Mitev, V. (2004). Simultaneous analysis of amino acid and biogenic polyamines by high-performance liquid chromatography after pre-column derivatization with N-(9-fluorenylmethoxycarbonyloxy)succinimide. *Journal of Chromatography. A*, *1025*(2), 201–208.

Lucas, P. M., Wolken, W. A. M., Claisse, O., Lolkema, J. S., & Lonvaud-Funel, A. (2005). Histamine-Producing Pathway Encoded on an Unstable Plasmid in Lactobacillus hilgardii 0006. *Applied and Environmental Microbiology*, *71*(3), 1417–1424.

Ludwig, I. A., Mena, P., Calani, L., Borges, G., Pereira-Caro, G., Bresciani, L., … Crozier, A. (2015). New insights into the bioavailability of red raspberry anthocyanins and ellagitannins. *Free Radical Biology and Medicine*, *89*, 758–769.

Ludwig, I. A., Paz de Peña, M., Concepción, C., & Alan, C. (2013). Catabolism of coffee chlorogenic acids by human colonic microbiota. *BioFactors*, *39*(6), 623–632.

Lyte, M. (2004). The biogenic amine tyramine modulates the adherence of Escherichia coli O157:H7 to intestinal mucosa. *Journal of Food Protection*, *67*(5), 878–883.

Magiera, S., Baranowska, I., & Kusa, J. (2012). Development and validation of UHPLC-ESI-MS/MS method for the determination of selected cardiovascular drugs, polyphenols and their metabolites in human urine. *Talanta*, *89*, 47–56.

Mahdy, A. M., & Webster, N. R. (2011). Histamine and antihistamines. *Anaesthesia & Intensive Care Medicine*, *12*, 324–329.

Maintz, L., & Novak, N. (2007). Histamine and histamine intolerance. *The American Journal of Clinical Nutrition*, *85*(5), 1185–1196.

Manach, C., Williamson, G., Morand, C., Scalbert, A., & Rémésy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *The American Journal of Clinical Nutrition*, *81*, 230–242.

Manzano, S., & Williamson, G. (2010). Polyphenols and phenolic acids from strawberry and apple decrease glucose uptake and transport by human intestinal Caco-2 cells. *Molecular Nutrition & Food Research*, *54*(12), 1773–1780.

Margalef, M., Pons, Z., Muguerza, B., & Arola-Arnal, A. (2014). A rapid method to determine colonic microbial metabolites derived from grape flavanols in rat plasma by liquid chromatography-tandem mass spectrometry. *Journal of Agricultural and Food Chemistry*, *62*, 7698–7706.

Martin, M. A., Goya, L., & Ramos, S. (2017). Protective effects of tea, red wine and cocoa in diabetes. Evidences from human studies. *Food and Chemical Toxicology*, *109*, 302–314.

Martínez-Pinilla, O., Guadalupe, Z., Hernández, Z., & Ayestarán, B. (2013). Amino acids and biogenic amines in red varietal wines: The role of grape variety, malolactic fermentation and vintage. *European Food Research and Technology*, *237*, 887–895.

Masella, R., Santangelo, C., D'Archivio, M., LiVolti, G., Giovannini, C., & Galvano, F. (2012). Protocatechuic Acid and Human Disease Prevention: Biological Activities and Molecular Mechanisms. *Current Medicinal Chemistry*, *19*(18), 2901–2917.

Masson, P. H., Takahashi, T., & Angelini, R. (2017). Editorial: Molecular Mechanisms Underlying Polyamine Functions in Plants. *Frontiers in Plant Science*, *8*, 1–3.

Mazzucco, E., Gosetti, F., Bobba, M., Marengo, E., Robotti, E., & Gennaro, M. C. (2010). High-performance liquid chromatography-ultraviolet detection method for the simultaneous determination of typical biogenic amines and precursor amino acids. applications in food chemistry. *Journal of Agricultural and Food Chemistry*, *58*, 127–134.

Mena, P., Calani, L., Bruni, R., & Del Rio, D. (2014). Bioactivation of High-Molecular-Weight Polyphenols by the Gut Microbiome. In *Diet-Microbe Interactions in the Gut: Effects on Human Health and Disease* (pp. 73–101). Elsevier.

Michael, A. J. (2016). Polyamines in eukaryotes, bacteria, and archaea. *Journal of Biological Chemistry*, *291*, 14896–14903.

Molenaar, D., Bosscher, J. S., Ten Brink, B., Driessen, A. J. M., & Konings, W. N. (1993). Generation of a proton motive force by histidine decarboxylation and electrogenic

histidine/histamine antiport in Lactobacillus buchneri. *Journal of Bacteriology*, *175*, 2864–2870.

Mosele, J. I., Macià, A., & Motilva, M.-J. (2016). Understanding of human metabolic pathways of different sub-classes of phenols from Arbutus unedo fruit after an acute intake. *Food & Function*, *7*, 1700–1710.

Muñoz-González, I., Jiménez-Girón, A., Martín-Álvarez, P. J., Bartolomé, B., & Moreno-Arribas, M. V. (2013). Profiling of microbial-derived phenolic metabolites in human feces after moderate red wine intake. *Journal of Agricultural and Food Chemistry*, *61*, 9470−9479.

Muñoz-González, I., Sánchez-Patán, F., Jiménez-Girón, A., Cueva, C., Monagas, M., Martín-Álvarez, P. J., … Bartolomé, B. (2014). Evaluation of SPE as Preparative Technique for the Analysis of Phenolic Metabolites in Human Feces. *Food Analytical Methods*, *7*, 844–853.

Nagy, K., Redeuil, K., Williamson, G., Rezzi, S., Dionisi, F., Longet, K., … Renouf, M. (2011). First identification of dimethoxycinnamic acids in human plasma after coffee intake by liquid chromatography–mass spectrometry. *Journal of Chromatography A*, *1218*(3), 491–497.

Naila, A., Flint, S., Fletcher, G., Bremer, P., & Meerdink, G. (2010). Control of Biogenic Amines in Food-Existing and Emerging Approaches. *Journal of Food Science*, *75*(7), R139–R150.

Nielsen, I. L. F., Chee, W. S. S., Poulsen, L., Offord-Cavin, E., Rasmussen, S. E., Frederiksen, H., … Williamson, G. (2006). Bioavailability is improved by enzymatic modification of the citrus flavonoid hesperidin in humans: a randomized, double-blind, crossover trial. *The Journal of Nutrition*, *136*(2), 404–408.

Nolvachai, Y., & Marriott, P. J. (2013). GC for flavonoids analysis: Past, current, and prospective trends. *Journal of Separation Science*, *36*, 20–36.

Nout, M. J. R. (1994). Fermented foods and food safety. *Food Research International*, *27*(3), 291–298.

Novotny, J. A., Clevidence, B. A., & Kurilich, A. C. (2012). Anthocyanin kinetics are dependent on anthocyanin structure. *British Journal of Nutrition*, *107*, 504–509.

Önal, A. (2007). A review: Current analytical methods for the determination of biogenic amines

in foods. *Food Chemistry*, *103*(4), 1475–1486.

Önal, A., Tekkeli, S. E. K., & Önal, C. (2013). A review of the liquid chromatographic methods for the determination of biogenic amines in foods. *Food Chemistry*, *138*(1), 509–515.

Opinion of the Scientific Panel on biological hazards (BIOHAZ) related to the effects of Nitrites/Nitrates on the Microbiological Safety of Meat Products. (2004). *EFSA Journal*, *2*(3), 14.

Ordóñez, J. L., Troncoso, A. M., García-Parrilla, M. D. C., & Callejón, R. M. (2016). Recent trends in the determination of biogenic amines in fermented beverages – A review. *Analytica Chimica Acta*, *939*, 10–25.

Ottaviani, J. I., Borges, G., Momma, T. Y., Spencer, J. P. E., Keen, C. L., Crozier, A., & Schroeter, H. (2016). The metabolome of [2-14C](-)-epicatechin in humans: Implications for the assessment of efficacy, safety, and mechanisms of action of polyphenolic bioactives. *Scientific Reports*, *6*, 29034.

Ou, K., & Gu, L. (2014). Absorption and metabolism of proanthocyanidins. *Journal of Functional Foods*, *7*, 43–53.

Ough, C. S., & Crowell, E. A. (1980). Nitrate determination in California musts and wines. *American Journal of Enology and Viticulture*, *31*(4), 344–346.

Ough, C. S., Daudt, C. E., & Crowell, E. A. (1981). Identification of new volatile amines in grapes and wines. *Journal of Agricultural and Food Chemistry*, *29*(5), 938–941.

Pacquit, A., Frisby, J., Diamond, D., Lau, K. T., Farrell, A., Quilty, B., & Diamond, D. (2007). Development of a smart packaging for the monitoring of fish spoilage. *Food Chemistry*, *102*, 466–470.

Patel, C. N., Wortham, B. W., Lines, J. L., Fetherston, J. D., Perry, R. D., & Oliveira, M. A. (2006). Polyamines are essential for the formation of plague biofilm. *Journal of Bacteriology*, *188*, 2355–2363.

Patrignani, F., Ndagijimana, M., Belletti, N., Gardini, F., Vernocchi, P., & Lanciotti, R. (2012). Biogenic Amines and Ethyl Carbamate in Primitivo Wine: Survey of Their Concentrations in Commercial Products and Relationship with the Use of Malolactic Starter. *Journal of Food Protection*, *75*(3), 591–596.

Paula Santos, N., Colaço, A., Gil da Costa, R. M., Manuel Oliveira, M., Peixoto, F., & Alexandra Oliveira, P. (2014). N-diethylnitrosamine mouse hepatotoxicity: Time-related

effects on histology and oxidative stress. *Experimental and Toxicologic Pathology*, *66*, 429–436.

Peña-Gallego, A., Hernández-Orte, P., Cacho, J., & Ferreira, V. (2009). Biogenic amine determination in wines using solid-phase extraction: A comparative study. *Journal of Chromatography A*, *1216*(15), 3398–3401.

Peña-Gallego, A., Hernández-Orte, P., Cacho, J., & Ferreira, V. (2012a). High-performance liquid chromatography analysis of amines in must and wine: a review. *Food Reviews International*, *28*(1), 71–96.

Pereira-Caro, G., Borges, G., Ky, I., Ribas, A., Calani, L., Del Rio, D., … Crozier, A. (2015). In vitro colonic catabolism of orange juice (poly)phenols. *Molecular Nutrition and Food Research*, *59*, 465–475.

Pereira-Caro, G., Borges, G., van der Hooft, J., Clifford, M. N., Del Rio, D., Lean, M. E., … Crozier, A. (2014). Orange juice (poly)phenols are highly bioavailable in humans. *American Journal of Clinical Nutrition*, *100*(5), 1378–1384.

Pereira-Caro, G., Ludwig, I. A., Polyviou, T., Malkova, D., García, A., Moreno-Rojas, J. M., & Crozier, A. (2016). Identification of Plasma and Urinary Metabolites and Catabolites Derived from Orange Juice (Poly)phenols: Analysis by High-Performance Liquid Chromatography-High-Resolution Mass Spectrometry. *Journal of Agricultural and Food Chemistry*, *64*, 5724−5735.

Pereira-Caro, G., Polyviou, T., Ludwig, I. A., Nastase, A. M., Moreno-Rojas, J. M., Garcia, A. L., ... & Crozier, A. (2017). Bioavailability of orange juice (poly) phenols: The impact of short-term cessation of training by male endurance athletes. *The American journal of clinical nutrition*, *106*(3), 791-800.

Pereira de Abreu, D. A., Cruz, J. M., & Paseiro Losada, P. (2012). Active and Intelligent Packaging for the Food Industry. *Food Reviews International* , *28*, 146–187.

Pérez-Magariño, S., Ortega-Heras, M., Martínez-Lapuente, L., Guadalupe, Z., & Ayestarán, B. (2013). Multivariate analysis for the differentiation of sparkling wines elaborated from autochthonous Spanish grape varieties: Volatile compounds, amino acids and biogenic amines. *European Food Research and Technology*, *236*, 827–841.

Petersen, J., Drasche, A., Raithel, M., & Schwelberger, H. G. (2003). Analysis of genetic polymorphisms of enzymes involved in histamine metabolism. *Inflammation Research*, *52*, S69–S70.

Porrini, M., & Riso, P. (2008). Factors influencing the bioavailability of antioxidants in foods: A critical appraisal. *Nutrition, Metabolism and Cardiovascular Diseases*, *18*(10), 647–650.

Prasain, J.K., Peng, N., Dai, Y., Moore, R., Arabshahi, A., Wilson, L., Barnes, S., Wyss, J.M., Kim, H., Watts, R.L. (2009). Liquid chromatography tandem mass spectrometry identification of proanthocyanidins in rat plasma after oral administration of grape seed extract. Phytomedicine, 16, 233-243.

Premont, R. T., Gainetdinov, R. R., & Caron, M. G. (2001). Following the trace of elusive amines. *Proceedings of the National Academy of Sciences of the United States of America*, *98*, 9474–9475.

Ramos, R. M., Valente, I. M., & Rodrigues, J. A. (2014). Analysis of biogenic amines in wines by salting-out assisted liquid-liquid extraction and high-performance liquid chromatography with fluorimetric detection. *Talanta*, *124*, 146–51.

Redeuil, K., Smarrito-Menozzi, C., Guy, P., Rezzi, S., Dionisi, F., Williamson, G., … Renouf, M. (2011). Identification of novel circulating coffee metabolites in human plasma by liquid chromatography-mass spectrometry. *Journal of Chromatography A*, *1218*, 4678–4688.

Redruello, B., Ladero, V., Cuesta, I., Álvarez-Buylla, J. R., Martín, M. C., Fernández, M., & Alvarez, M. A. (2013). A fast, reliable, ultra high performance liquid chromatography method for the simultaneous determination of amino acids, biogenic amines and ammonium ions in cheese, using diethyl ethoxymethylenemalonate as a derivatising agent. *Food Chemistry*, *139*, 1029–1035.

Reglamento (CE) Nº 1924/2006 del Parlamento Europeo y del Consejo. (2006).

Rhee, J. E., Rhee, J. H., Ryu, P. Y., & Choi, S. H. (2002). Identification of the cadBA operon from Vibrio vulnificus and its influence on survival to acid stress. *FEMS Microbiology Letters*, *208*(2), 245–251.

Rodríguez-Kessler, M., Ruiz, O. A., Maiale, S., Ruiz-Herrera, J., & Jiménez-Bremont, J. F. (2008). Polyamine metabolism in maize tumors induced by Ustilago maydis. *Plant Physiology and Biochemistry*, *46*, 805–814.

Rodriguez-Mateos, A., Vauzour, D., Krueger, C. G., Shanmuganayagam, D., Reed, J., Calani, L., … Crozier, A. (2014). Bioavailability, bioactivity and impact on health of dietary flavonoids and related compounds: an update. *Archives of Toxicology*, *88*, 1803–1853.

Rodriguez-Naranjo, M. I., Ordóñez, J. L., Callejón, R. M., Cantos-Villar, E., & Garcia-Parrilla, M. C. (2013). Melatonin is formed during winemaking at safe levels of biogenic amines. *Food and Chemical Toxicology*, *57*, 140–146.

Roowi, S., Stalmach, A., Mullen, W., Lean, M. E. J., Edwards And, C. A., & Crozier, A. (2010). Green tea flavan-3-ols: Colonic degradation and urinary excretion of catabolites by humans. *Journal of Agricultural and Food Chemistry*, *58*, 1296–1304.

Russo, P., Fernández de Palencia, P., Romano, A., Fernández, M., Lucas, P., Spano, G., & López, P. (2012). Biogenic amine production by the wine Lactobacillus brevis IOEB 9809 in systems that partially mimic the gastrointestinal tract stress. *BMC Microbiology*, *12:247*, 1–10.

Saenger, T., Hübner, F., & Humpf, H. U. (2017). Short-term biomarkers of apple consumption. *Molecular Nutrition and Food Research*, *61*, 1–10.

Sano, A., Yamakoshi, J., Tokutake, S., Tobe, K., Kubota, Y., Kikuchi, M. Procyanidin B1 is detected in human serum after intake of proanthocyanidin-rich grape extract (2003). *Bioscience, biotechnology, and biochemistry*, 1140-1143.

Saha, S., Hollands, W., Needs, P. W., Ostertag, L. M., de Roos, B., Duthie, G. G., & Kroon, P. A. (2012). Human O-sulfated metabolites of (−)-epicatechin and methyl-(−)-epicatechin are poor substrates for commercial aryl-sulfatases: Implications for studies concerned with quantifying epicatechin bioavailability. *Pharmacological Research*, *65*(6), 592–602.

Sánchez-Jiménez, F., Ruiz-Pérez, M. V, Urdiales, J. L., & Medina, M. A. (2013). Pharmacological potential of biogenic amine-polyamine interactions beyond neurotransmission. *British Journal of Pharmacology*, *170*(1), 4–16.

Sánchez-Patán, F., Monagas, M., Moreno-Arribas, M. V., & Bartolomé, B. (2011). Determination of microbial phenolic acids in human faeces by UPLC-ESI-TQ MS. *Journal of Agricultural and Food Chemistry*, *59*, 2241–2247.

Sasot, G., Martínez-Huélamo, M., Vallverdú-Queralt, A., Mercader-Martí, M., Estruch, R., & Lamuela-Raventós, R. M. (2017). Identification of phenolic metabolites in human urine after the intake of a functional food made from grape extract by a high resolution LTQ-Orbitrap-MS approach. *Food Research International*, *100*, 435–444.

Savage, A. K., Duynhoven, J. P. M., Tucker, G., & Daykin, C. A. (2011). Enhanced NMR-based profiling of polyphenols in commercially available grape juices using solid-phase extraction. *Magnetic Resonance in Chemistry*, *49*(S1), S27–S36.

Scalbert, A., Manach, C., Morand, C., Rémésy, C., & Jiménez, L. (2005). Dietary Polyphenols and the Prevention of Diseases. *Critical Reviews in Food Science and Nutrition*, *45*(4), 287–306.

Scalbert, A., Morand, C., Manach, C., & Rémésy, C. (2002). Absorption and metabolism of polyphenols in the gut and impact on health. *Biomedicine & Pharmacotherapy*, *56*, 276–282.

Scalbert, A., & Williamson, G. (2000). Dietary intake and bioavailability of polyphenols. *The Journal of Nutrition*, *130*(8S), 2073S–2085S.

Schwelberger, H. G., Drasche, A., Petersen, J., & Raithel, M. (2003). Genetic polymorphisms of histamine degrading enzymes: From small-scale screening to high-throughput routine testing. *Inflammation Research*, *52*, S71–S73.

Scientific Opinion on risk based control of biogenic amine formation in fermented foods EFSA Panel on Biological Hazards (BIOHAZ). (2011). *EFSA Journal*, *9993*(1010).

Scientific Opinion on the substantiation of a health claim related to cocoa flavanols and maintenance of normal endothelium-dependent vasodilation pursuant to Article 13(5) of Regulation (EC) No 1924/2006. (2012). *EFSA Journal*, *10*(7).

Scientific Opinion on the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage (ID 1333, 1638, 1639, 1696, 2865), maintenance of normal blood HDL cholesterol concentrations (ID 1639), mainte. (2011). *EFSA Journal*, *9*(4), 2033.

Selma, M. V., Espín, J. C., & Tomás-Barberán, F. A. (2009). Interaction between Phenolics and Gut Microbiota: Role in Human Health. *Journal of Agricultural and Food Chemistry*, *57*(15), 6485–6501.

Sempruch, C., Leszczyński, B., Wilczewska, M., Chrzanowski, G., Sytykiewicz, H., Goławska, S., … Klewek, A. (2015). Changes in amino acid decarboxylation in maize (Zea mays; Poaceae) tissues in response to bird cherry-oat aphid (Rhopalosiphum padi; Aphididae) infestation. *Biochemical Systematics and Ecology*, *60*, 158–164.

Sentellas, S., Núñez, Ó., & Saurina, J. (2016). Recent Advances in the Determination of Biogenic Amines in Food Samples by (U)HPLC. *Journal of Agricultural and Food Chemistry*, *64*(41), 7667–7678.

Serra, A., Macià, A., Romero, M. P., Piñol, C., & Motilva, M. J. (2011). Rapid methods to

determine procyanidins, anthocyanins, theobromine and caffeine in rat tissues by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *879*, 1519–1528.

Shalaby, A. R. (1996). Significance of biogenic amines to food safety and human health. *Food Research International*, *29*(7), 675–690.

Silla-Santos, M. H. (1996). Biogenic amines: their importance in foods. *International Journal of Food Microbiology*, *29*(2–3), 213–231.

Sirocchi, V., Caprioli, G., Cecchini, C., Coman, M. M., Cresci, A., Maggi, F., … Sagratini, G. (2013). Biogenic amines as freshness index of meat wrapped in a new active packaging system formulated with essential oils of Rosmarinus officinalis. *International Journal of Food Sciences and Nutrition*, *64*(8), 921–928.

Soda, K. (2011). The mechanisms by which polyamines accelerate tumor spread. *Journal of Experimental & Clinical Cancer Research*, *30*(95), 1–9.

Spano, G., Russo, P., Lonvaud-Funel, A., Lucas, P., Alexandre, H., Grandvalet, C., … Lolkema, J. S. (2010). Biogenic amines in fermented foods. *European Journal of Clinical Nutrition*, *64*, 95–100.

Spencer, J. P. E. (2003). Metabolism of tea flavonoids in the gastrointestinal tract. *The Journal of Nutrition*, *133*(10), 3255S–3261S.

Stalmach, A., Mullen, W., Barron, D., Uchida, K., Yokota, T., Cavin, C., … Crozier, A. (2009). Metabolite profiling of hydroxycinnamate derivatives in plasma and urine after the ingestion of coffee by humans: Identification of biomarkers of coffee consumption. *Drug Metabolism and Disposition*, *37*, 1749–1758.

Stalmach, A., Mullen, W., Steiling, H., Williamson, G., Lean, M. E. J., & Crozier, A. (2010). Absorption, metabolism, and excretion of green tea flavan-3-ols in humans with an ileostomy. *Molecular Nutrition and Food Research*, *54*, 323–334.

Stockley, C., Teissedre, P.-L., Boban, M., Di Lorenzo, C., & Restani, P. (2012). Bioavailability of wine-derived phenolic compounds in humans: a review. *Food & Function*, *3*(10), 995–1007.

Stoupi, S., Williamson, G., Drynan, J. W., Barron, D., & Clifford, M. N. (2010a). A comparison of the in vitro biotransformation of (-)-epicatechin and procyanidin B2 by human faecal microbiota. *Molecular Nutrition and Food Research*, *54*, 747–759.

Stoupi, S., Williamson, G., Drynan, J. W., Barron, D., & Clifford, M. N. (2010b). Procyanidin B2 catabolism by human fecal microflora: Partial characterization of "dimeric" intermediates. *Archives of Biochemistry and Biophysics*, *501*, 73–78.

Stoupi, S., Williamson, G., Viton, F., Barron, D., King, L. J., Brown, J. E., & Clifford, M. N. (2010). In vivo bioavailability, absorption, excretion, and pharmacokinetics of [14C]procyanidin B2 in male rats. *Drug Metabolism and Disposition*, *38*, 287–291.

Strack, D., & Wray, V. (1989). Anthocyanins. *Methods in Plant Biochemistry*, *1*, 325–356.

Stratton, J. E., Hutkins, R. W., & Taylor, S. L. (1991). Biogenic Amines in Cheese and other Fermented Foods: A Review. *Journal of Food Protection*, *54*(6), 460–470.

Taverniers, I., De Loose, M., & Van Bockstaele, E. (2004). Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance. *TrAC - Trends in Analytical Chemistry*, *23*, 535–552.

Taylor, S. L., & Eitenmiller, R. R. (1986). Histamine Food Poisoning: Toxicology and Clinical Aspects. *CRC Critical Reviews in Toxicology*, *17*(2), 91–128.

ten Brink, B., Damink, C., Joosten, H., & Huis In't Veld, J. H. J. (1990). Occurrence and formation of biologically active amines in foods. *International Journal of Food Microbiology*, *11*(1), 73–84.

Til, H. P., Falke, H. E., Prinsen, M. K., & Willems, M. I. (1997). Acute and subacute toxicity of tyramine, spermidine, spermine, putrescine and cadaverine in rats. *Food and Chemical Toxicology*, *35*, 337–348.

Tkachenko, A., Nesterova, L., & Pshenichnov, M. (2001). The role of the natural polyamine putrescine in defense against oxidative stress in Escherichia coli. *Archives of Microbiology*, *176*(1–2), 155–157.

Tsang, C.; Auger, C.; Mullen, W.; Bornet, A.; Rouanet, J. M.; Crozier, A.; Teissedre, P. L. (2005). The absorption, metabolism and excretion of flavan-3-ols and procyanidins following the ingestion of a grape seed extract by rats. *British Journal of Nutrition*, *94*, 170–181.

Tuberoso, C. I. G., Congiu, F., Serreli, G., & Mameli, S. (2015). Determination of dansylated amino acids and biogenic amines in Cannonau and Vermentino wines by HPLC-FLD. *Food Chemistry*, *175*, 29–35.

Urpi-Sarda, M., Monagas, M., Khan, N., Lamuela-Raventos, R. M., Santos-Buelga, C.,

Sacanella, E., … Andres-Lacueva, C. (2009). Epicatechin, procyanidins, and phenolic microbial metabolites after cocoa intake in humans and rats. *Analytical and Bioanalytical Chemistry*, *394*, 1545–1556.

Urpi-Sarda, M., Rothwell, J., Morand, C., & Manach, C. (2012). Bioavailability of flavanones. In A. C. JPE Spencer (Ed.), *Bioavailability and Function of Flavonoids* (pp. 1–44).

Valdés-Santiago, L., Cervantes-Chávez, J. A., León-Ramírez, C. G., & Ruiz-Herrera, J. (2012). Polyamine Metabolism in Fungi with Emphasis on Phytopathogenic Species. *Journal of Amino Acids*, *2012*, 1–13.

Valdés-Santiago, L., & Ruiz-Herrera, J. (2014). Stress and polyamine metabolism in fungi. *Frontiers in Chemistry*, *1*, 1–10.

van de Wetering, K., Burkon, A., Feddema, W., Bot, A., de Jonge, H., Somoza, V., & Borst, P. (2009). Intestinal Breast Cancer Resistance Protein (BCRP)/Bcrp1 and Multidrug Resistance Protein 3 (MRP3)/Mrp3 Are Involved in the Pharmacokinetics of Resveratrol. *Molecular Pharmacology*, *75*(4), 876–885.

van der Hooft, J. J. J., de Vos, R. C. H., Mihaleva, V., Bino, R. J., Ridder, L., de Roo, N., … Vervoort, J. (2012). Structural elucidation and quantification of phenolic conjugates present in human urine after tea intake. *Analytical Chemistry*, *84*, 7263–7271.

Vauzour, D. (2012). Dietary polyphenols as modulators of brain functions: Biological actions and molecular mechanisms underpinning their beneficial effects. *Oxidative Medicine and Cellular Longevity*, *2012*, 1–16.

Verzelloni, E., Pellacani, C., Tagliazucchi, D., Tagliaferri, S., Calani, L., Costa, L. G., … Del Rio, D. (2011). Antiglycative and neuroprotective activity of colon-derived polyphenol catabolites. *Molecular Nutrition & Food Research*, *55*(S1), S35–S43.

Walters, D. R. (2003). Polyamines and plant disease. *Phytochemistry*, *64*(1), 97–107.

Wang, Y.-Q., Ye, D.-Q., Zhu, B.-Q., Wu, G.-F., & Duan, C.-Q. (2014). Rapid HPLC analysis of amino acids and biogenic amines in wines during fermentation and evaluation of matrix effect. *Food Chemistry*, *163*, 6–15.

Weichselbaum, E., & Buttriss, J. L. (2010). Polyphenols in the diet. *Nutrition Bulletin*, *35*, 157–164.

Williamson, G., & Clifford, M. N. (2017). Role of the small intestine, colon and microbiota in determining the metabolic fate of polyphenols. *Biochemical Pharmacology*, *139*, 24–39.

Wolken, W. A. M., Lucas, P. M., Lonvaud-Funel, A., & Lolkema, J. S. (2006). The mechanism of the tyrosine transporter TyrP supports a proton motive tyrosine decarboxylation pathway in Lactobacillus brevis. *Journal of Bacteriology*, *188*, 2198–2206.

Xiao, Y., Hu, Z., Yin, Z., Zhou, Y., Liu, T., Zhou, X., & Chang, D. (2017). Profiling and distribution of metabolites of procyanidin B2 in mice by UPLC-DAD-ESI-IT-TOF-MSn technique. *Frontiers in Pharmacology*, *8*, 1–12.

Xie, L., Lee, S. G., Vance, T. M., Wang, Y., Kim, B., Lee, J. Y., … Bolling, B. W. (2016). Bioavailability of anthocyanins and colonic polyphenol metabolites following consumption of aronia berry extract. *Food Chemistry*, *211*, 860–868.

Zhang, M., Jagdmann, G. E., Van Zandt, M., Sheeler, R., Beckett, P., & Schroeter, H. (2013). Chemical Synthesis and Characterization of Epicatechin Glucuronides and Sulfates: Bioanalytical Standards for Epicatechin Metabolite Identification. *Journal of Natural Products*, *76*(2), 157–169.

Zimatkin, S. M., & Anichtchik, O. V. (1999). Alcohol-histamine interactions. *Alcohol and Alcoholism (Oxford, Oxfordshire)*, *34*(2), 141–147.